The causes and costs of intersexuality in two freshwater populations of the amphipod, *Gammarus minus* found in Montgomery County, Virginia USA

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Abstract

Intersexuality, the abnormal condition whereby gonochoristic animals display characteristics of both males and females, is common but not overly prevalent amongst the animal kingdom. However, females of the amphipod *Gammarus minus* inhabiting two freshwater springs in Virginia (USA) exhibit unusually high (60 – 100%) frequencies of intersexuality. The overall aim of my study is to explain these unusually high frequencies by identifying potential cause(s) and reproductive costs of intersexuality in these two study populations. Sex determination mechanisms in Crustacea are complex but, what is known is that sex determination can be influenced by any or all of the following mechanisms, parasitic infections, genetics (i.e. polyfactorial systems), and environmental factors including anthropogenic disruption, all of which may occur singularly or simultaneously within a species.

Currently known causes of intersexuality are considered appropriate starting points to evaluate these two populations. The effect of endocrine disruption is evident within reproductive development of the following: fecundity, fertility, growth, maturation and development, pre-copular behavior and atypical phenotypes. I employed field methods to evaluate the effect of intersexuality on population dynamics and laboratory methods to investigate the prevalence of parasites and costs of the intersex condition. Using the data from my field and laboratory investigation, the findings suggest that sex ratios may be driven by photoperiod. In both populations, the expectation that the cost associated with female intersexuality effects reproductive fitness is accurate, when compared with normal female populations.

Intersex specimen's body lengths were consistently larger than their respective sexual phenotypes, and intersex females produced 29% fewer eggs than normal females. Intersex females matured at a greater size than normal females indicating a delay to maturity, which was verified by growth and development patterns between intersex and normal females. Normal female patterns for growth, maturation, development and fecundity were continuous with no obvious pattern, while intersex females from both populations are similar in a pattern that peaks early autumn to winter. Contrary to my expectation, pre-copular behavioral experiments regarding the costs of

intersexuality of normal versus intersex females revealed that males had no preference for normal females over intersex females. However, when males that are originally paired with intersex females are separated, it takes twice as long to rejoin when compared with the normal females and males.

The findings of this study offer insights into sex determination and the possible cause of intersexuality of these two populations, as well as reproductive costs. The choice of observations regarding the effects of intersexuality (fecundity, fertility, growth, maturation and pre-copular behavioral experiments) was appropriate for evaluating the effect and costs of the intersex condition when compared with normal females. Regardless, more molecular methods are needed to identify the newly revealed microsporidian parasite. While the underlying cause of the intersex condition in both populations suggests photoperiod, it is also likely that the populations are influenced by a novel feminizing parasite. The unique prevalence of intersex *G. minus* allows great opportunities to explore the avenues of known causes of intersexuality, as well as unknown causes.

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Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are my own and have not been submitted for any other academic award.

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Abbreviations

ESD	environmental sex determination
EDC	endocrine disruption contaminant
GSD	genetic sex determination
PSD	parasite sex determination

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1. Introduction

1.1 Intersexuality

The term intersexuality was coined by Richard Goldschmidt in 1917. Initially, it was intended for the definition to apply to a normally dioecious species that exhibited a mixture between male and female (Goldschmidt, 1917). Subsequent to Goldschmidt, intersex specimens were observed in the amphipod *G. cheureuxi* by Huxley (1921) and Sexton (1924) who initially gave physical descriptions of the intersex organisms. Later in 1931 and after conducting a comprehensive study of sex determination (in *Aphyosemion punctatum*), Goldschmidt reported the first evidence of intersex in fish. Since then, the definition of intersex has changed as knowledge and prevalence of the intersex condition has increased (Bahamonde et al., 2013). In more detail, Gomes et al., (2009) described intersexuality as a condition whereby a gonochoristic individual has oocytes or distinct stages of spermatogonia, with varying degrees of development within the gonad of the opposite gender (i.e. spermatocytes in the ovary or oocytes in the testis.). Stentiford (2012) further added that changes may be restricted to the external morphology or to internal alterations in the structure and function of the sex organs that would not necessarily display both simultaneously (Stentiford, 2012). The expression of intersexuality may differ from one organism to another; however the basic characterization is rooted in organisms presenting both male and female characteristics (Reinboth, 1975).

Intersexuality has been widely reported across the animal kingdom including crustacean populations (Dunn et al., 1990; Glazier et al., 2012; Ford and Fernandes, 2005). Intersexuality is documented in both wild and laboratory animals, from aquatic invertebrates to vertebrates including fish, amphibians (Hecker et al., 2006), reptiles (Forbes, 1962) and birds (Gomot, 1975). There are approximately 340 aquatic invertebrate species that display intersexuality. Within the two phyla Mollusca and Arthropoda, the prevalence of species that exhibit intersexuality breaks down as follows: Molluscs ~268 (Gastropoda 256, Bivalvia 7, Cephalopoda 2, Polyplacophora 1) and Arthropods ~71 (Decopoda 22, Copepoda 20, Amphipoda 20, Cladocera 3, Mysidacea 3, Isopoda 2, Anostraca 1).

1.2 Intersexuality in amphipods

Intersexuality has been observed in many amphipods, for example within the genera *Gammarus*, intersex individuals have been recorded from 0.1 to 100% of total populations (Glazier et al., 2009; Miller, 1977; Buikema, 1980) with varying degrees of prevalence. The mechanisms of sex determination are multi-faceted (i.e. genetic, environmental, anthropogenic, and geographical), however the specific gland ultimately responsible was first discovered in 1947 by Cronin. The multiple variables mentioned above, have great influence on sex determination and how this gland is or is not, activated.

The androgenic gland (AG) was first discovered (by Cronin in 1947) in the decapod crustacean, the crab *Callinectes sapidus*, subsequent to Cronin's discovery was verification of the AG's connection in male differentiation and spermatogenesis in the amphipod (Charniaux-Cotton, 1954). However, the association of the intersex phenomena and endocrine disruption in crustaceans was first linked by Khalaila et al., (2001). Studies have supported this association and multiple factors are believed to be involved; such as, the disruption of the androgenic gland (AG) (Charniaux-Cotton, 1958); the presence of sex distorting parasites (Rodgers-Gray et al., 2004), environmental sex determination (ESD) (Dunn et al., 1996) and chemical pollution (Ford et al., 2006; Olmstead and LeBlanc, 2007). If the AG is disrupted by any of these factors, the AG hormone (AGH) is reduced and results in de-masculinisation, cessation of spermatogenesis and even in some cases, ovarian development (Barki et al., 2006). Further clarification of ESD follows and includes factors such as, environmental parameters, anthropogenic factors, as well as cytoplasmic influence. Considering embryonic development may be determined by these various cues (i.e. stream temperatures, nutrients, pollutants, and photoperiod), the incidence of simultaneous influence is frequent (Dunn et al., 1996).

ESD is the establishment of sex by various non-genetic cues, which is in contrast with genotypic sex determination (GSD), because sex is not determined at conception but at a discrete period after (Bull, 1983). ESD is very widespread throughout the animal kingdom and has been documented in diverse groups of organisms, for example, Echiura, reptiles, fish, nematodes and crustaceans (Adams et al., 1987; Korpelainen,

1990; Ciofi and Swingland, 1995; Conover and Kynard, 1961; Bull, 1980; and Petersen, 1972). In Charnov and Bull (1977), the proposed model is that ESD is favoured by natural selection when an individual's fitness is strongly influenced by environmental conditions, and when that individual has little control over its environment (i.e. invertebrates in aquatic systems). Their proposed model, also accounts for adaptiveness, which is often thought of as an evolutionary mechanism (Charnov and Bull, 1977). The underlying mechanism of ESD, natural selection, has two possible disadvantages (Bull, 1985a); one, sex is determined late in development in response to environmental conditions, thus, intersexes may result. Secondly, the influence of climatic changes (e.g. climate change, anthropogenic causes) allows influence on the populations sex ratios (e.g. male-biased populations). Of the various sex-determining factors, temperature is most widely studied, particularly in reptiles. For example, in turtles (i.e. painted (*Chrysemys picta*) and snapping turtles (*Chelydra serpentine*) the incubation temperature during embryogenesis has been shown to control sex (Wilhoft et al., 1983). In the study conducted by Wilhoft et al. (1983), it was found that warm temperatures (30^{o}C) ensured female development and intermediate temperatures (22 - 28^{o}C) resulted in male development. According to Janzen and Paukstis (1991), this manner of development occurs during the middle trimester of embryonic development. Sex expression in these species is known as temperature-dependent sex determination (TSD) (Gutzke and Paukstis, 1983; Packard et al., 1987; Witschi, 1930).

In Crustacea, the basis of sex determination is genetic, although various environmental and cytoplasmic influences have been shown in some species (Legrand, Legrand-Hamelin and Juchault, 1987). In aquatic systems, the sex-determining factors temperature and photoperiod are most important; however other influences include, but are not limited to, nutrition, population density, pH, carbon dioxide, pollutants, metabolic products, and cytoplasmic factors (detailed in Chapter 4) (Korpelainen, 1990). For example, the crustacean *Daphnia magna* has the ability to switch from parthenogenic (cloning) to sexual reproduction when environmental conditions dictate (Herbert, 1978). Under favourable conditions females are increased and undergo parthenogenesis, while in unfavourable conditions (i.e. reduced light period and diet) males are increased and sexual reproduction occurs (Hebert, 1978).

The amphipod *G. duebeni*, found in brackish waters, exhibits seasonally sex biased ratios as influenced by photoperiod (Bulnheim, 1966, 1978; Nayor et al., 1988a). However, field and laboratory studies did not correlate (Watt and Adams, 1994) and so, this indicated that a second environmental cue was likely influencing sex determination of *G. duebeni* (Watt and Adams, 1993). Since then, Dunn et al., (2005) conducted research on *G. duebeni*, and determined that photoperiod and temperatures are both environmental cues for this species. In Dunn et al. (2005) four U.K populations, which were geographically separated (two northern sites and two southern sites) showed significant variance between the separate populations; the two northern sites had male biased broods under long day warm conditions and female biased broods under short day warm regimes, but during cold conditions sex ratios would reverse. The two southern populations were only influenced by photoperiod, resulting in male biased broods under long day conditions. In addition, the Dunn et al., (2005) study, reveals that adaptive variation occurs within *G. duebeni*.

Endocrine disruption and the possible impacts on vertebrate health have become an active area of research since a wide variety of exogenous chemicals were linked to harmful effects on endocrine systems, and that endocrine disruption contaminants (EDC's) involve the estrogenic receptor (United States Environmental Protection Agency (US EPA), 2018, 1997; European Community 1997; Kavlock et al., 1996; Degen and Bolt, 2000). Among some of the most ubiquitous EDC's are estrogen mimics (Colborn, 1996). A wide range of substances (e.g. pharmaceuticals, dioxin and dioxin-compounds, polychlorinated biphenyls, DDT and other pesticides, and bisphenol A) that may be found in many everyday products (including detergents, plastic bottles, food, toys, metal food cans, and pesticides) have been identified as possible EDC's (National Institute of Environmental Health Sciences, 2019).

Significant research on endocrine systems and EDC's has increased our knowledge of hormonal effects on vertebrate systems (Degen and Bolt, 2000). For example, from the 1940's to 1970's, the pharmaceutical diethylstilbestrol (DES) was used to prevent miscarriage, however years later (1972), DES was linked to a rare form of vaginal cancer in daughters whose mothers received DES (Colborn, 1996). Conversely, research on invertebrates and EDC's has not progressed at the same rate. Particularly understudied, is the disruption of the endocrine system pathways (e.g. EDCysone

receptor-mediated signalling) and chemical contaminants, which are responsible for crustacean growth, development and reproduction (Colborn, 1996; Short et al., 2014). Although our knowledge of environmental contaminants remains incomplete, a frame of reference for the classification of xenobiotics (naturally occurring) and anthropogenic pollutants (i.e. chemical pollution) have been acknowledge (United States Environmental Protection Agency (US EPA), 2018; Colborn, 1996).

Crustacean's present nonparasitic and parasitic-induced forms of intersexuality, which are associated with environmental contaminates (Ford et al., 2004; Short et al., 2014). Evidence that exogenous chemicals may disrupt normal endocrine function has become more prevalent than ever before, and this has raised concern that environmental contaminants may be affecting the AG (Ford et al., 2004). Heavy metals (e.g. nickel, copper, zinc, and lead) and waste by-products (e.g. acid wastes) have become prevalent in the aquatic ecosystems due to various industry discharges (e.g. mining, industry, power plants, and waste water/sewage treatment plants). Discharges may be illegally dumped into a waste stream, or if it is legal, the standard for the pollutant may be too lenient (United States Environmental Protection Agency (US EPA), 2018, 1997). Freshwater and marine habitats are vulnerable to many types of pollutant sources, which in turn, expose aquatic invertebrates (i.e. amphipods) to a variety of contaminants.

For the first time, the occurrence of intersexuality in the porcelain crabs *Lissoporcellana quadrilobata*, *Pisidia bluteli*, and *Pisidia longimana* were found in highly polluted coastal regions (Ferreira, 2018). Coastal areas are typically polluted from petroleum products released during activities of coastal industries, lighthouses, harbours and boating (Ferreira, 2018). Individual porcelain crabs were sexed based upon the position of the gonopores and secondary sexual characters (males = modified pleopods on the second abdominal somite, and females = three pairs of uniramous pleopods located on the third, fourth and fifth abdominal somite). In normal females gonopores are located on the third perepods (P3), on normal males' gonopores are located on the coxae of the fifth perepods (P5), and on intersexes gonopore openings are located on both P3 and P5 of a single individual. Ferreira's (2018) examinations revealed that of the collected specimens the following numbers were intersexes, *L. quadrilobata* 2 of 23, *P. bluteli* 5 of 16, and *P. longiuana* 4 of 300. The frequency of

intersexes found in known polluted habitats, led Ferreira (2018) to hypothesize the occurrence is due to pollution or genetic abnormality (Ginsburger-Vogel and Charniaux-Cotton, 1982).

Bisphenol A (BPA), has received considerable attention regarding exposure and the effect on the endocrine system (Damstra et al. 2002). It has been established that BPA is an EDC, which has estrogenic activity and influence on reproductive systems and embryonic development in aquatic invertebrates (Zucker & Johnson, 1985). An interesting study by Li et al., (2018) looked at the effects of BPA on the expression of five genes (MnMago, MnTsu, MnGus, MnUbc9 and MnvWD-Kazal) responsible for ovary development in the oriental river prawn (*Macrobrachium nipponense*). Five exposure levels (5.01, 7.76, 12.06, 18.62, and 28.84 mg/l) were utilized with 96 prawns for 19 days; subsequent to exposures ovary tissues were removed and collected for analysis. The overall results showed that expression profiles of ovary genes significantly changed along with the exposure gradient, but that also a BPA concentration under 5.01 mg/L might be safe, however exceeding 12.06 mg/L may be harmful (Li et al., 2018). The scarcity of research on aquatic invertebrate (e.g. amphipods) is partly due to the complexities of the endocrine system and the precise mechanisms involved. Further investigation is essential to ascertain if amphipods are affected by the various environmental cues.

Intersexes in *G. minus* (males and females) are both larger in size than the normal phenotype of each organism (Miller, 1977; Ford and Glazier, 2008; Glazier et al., 2012). Intersex females can be female (with a brood) and also possess male secondary sexual characteristics, such as one or two papillae (Figure 1); intersex males can be genetically male and possess female secondary sexual characteristics, such as rudimentary brood plates, as well as, primary sex characteristics (i.e. an oviduct structure) (Ford et al., 2008).

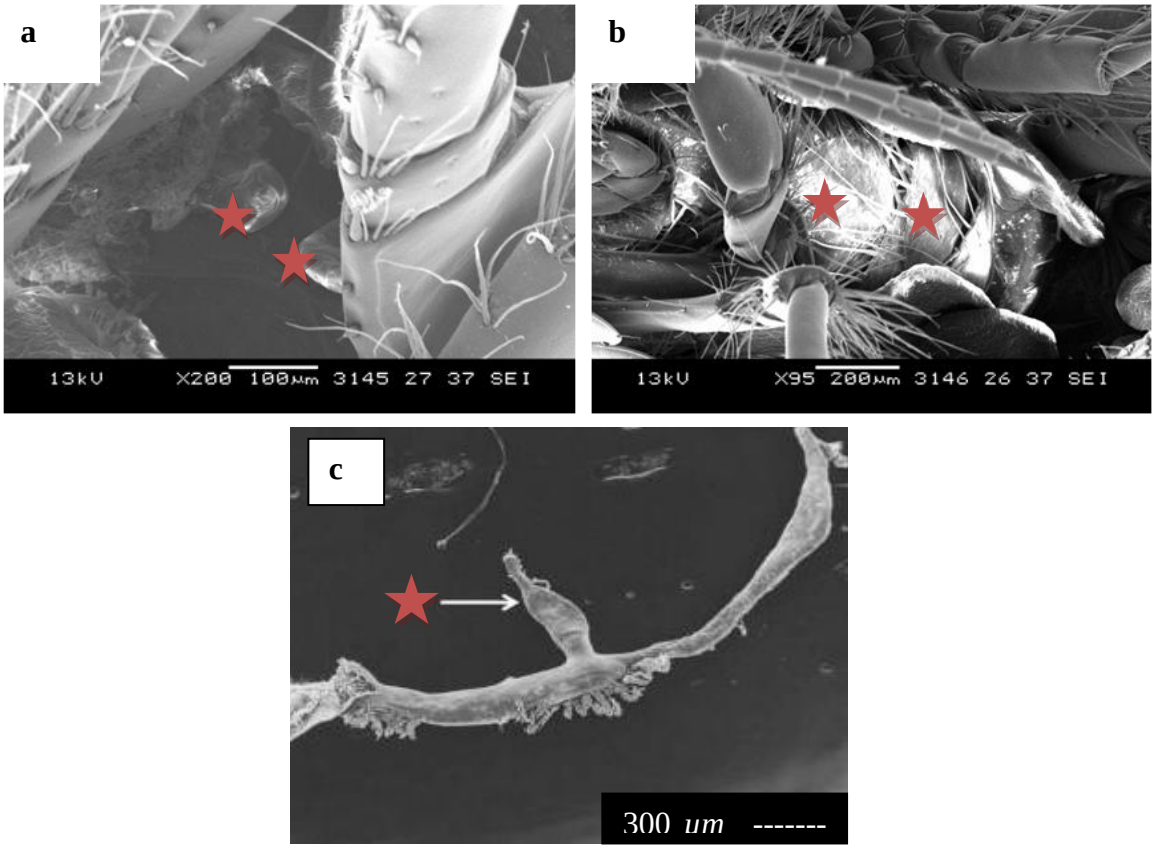

Figure 1. (a) FBR *G. minus* intersex female with two papillae (red stars), (b) Same intersex female carrying brood and presenting matured brood plates (red stars), (c) Internally intersex Echinogammarus marinus displaying testes with an oviduct (red star) taken from Ford et al., (2008). (University of Portsmouth, Portsmouth UK, 2012).

A number of costs have been reported with intersex amphipods. Ford et al., (2004) suggested that intersexes mature and grow larger in size due to a shift in energy from reproductive development to somatic growth. Additionally, fecundity, fertility, embryonic survival and mass, as well as, delayed maturation have been documented (Ford et al., 2003, 2004). Ford et al. (2003a) documented that the marine amphipod *E. marinus* intersex females produced ~20% less eggs than normal females and 10% reduced fecundity. Ford et al. (2003a) theorized that the possible reason for loss of eggs and/or embryos may be due to non-viable eggs being ejected or passively lost due to malformed brood plates. The Ford (2003) study also showed a variable cost of intersexuality regarding fecundity and fertility that was correlated with a degree of intersexuality (i.e. whether intersex females had one or two papillae). Reproductive costs cited by Ford (2003) included differences between papilla numbers (1 or 2) in females, for example, one-papilla females brood sizes were variable and two-papilla females tended to lose more eggs and/or embryos from the brood pouch (36%); in addition, Dunn et al. (1993) found that *G. duebeni* intersex females are known to have testicular tissue that may affect ovarian development. Furthermore, it is theorized that intersex females may possibly possess a rudimentary androgen gland, which might secret androgenic gland hormones that have also been shown to suppress ovarian development (Taketoni and Nishikawa, 1996). In breeding experiments by Sexton (1924), intersex *G. cheureuxi* males all took longer to reach maturity than normal males, which was also given as cause for the increased size of intersex verses normal animals (i.e. delayed maturation).

Gnathopod size is clearly a morphological characteristic that helps discriminate between male and female amphipods (Darwin, 1871), especially in species that carry the female in a precopular guarding phase. Posterior gnathopods have been shown in the Hume et al., (2005) study on *G. pulex* to support the role of precopular guarding. The study considered various functions of the posterior gnathopods; stimulating female moult, egg development, male-to-male fighting and subduing females were all tested by ablation experiments. The results concluded the function of the posterior gnathopods was to facilitate copulation; ablation of the gnathopods revealed that they were not necessary for mate guarding, however only males with intact gnathopods were able to successfully copulate (Hume et al., 2005). Intersex males' gnathopods are smaller than normal males' gnathopods; this could be a reproductive costs

considering it was shown by Hume (2005) that gnathopods were required for copulation. Not only is gnathopod size a required component of copulation, but the male is also known to allocate sperm based on parasitically infected or uninfected females (Dunn et al., 2005).

As cited by Miller (1977), Glazier and Ford (2008), and Glazier et al., (2012), intersex females produce smaller numbers of broods than normal females regardless of the cause of intersexuality (e.g. cytoplasmic parasites, ESD and pollution). Several studies suggest the male gender has the ability to allocate sperm to females that are prone to increased fecundity, hence normal, uninfected females (Rigaud and Moreau, 2004; Dunn et al., 2005). Dunn et al. (2005) conducted experiments to verify that *G. duebeni* did allocate fewer sperm to females infected with the parasite *Nosema granulosis* (microsporidia spp); considering infected females are strongly and positively correlated with intersexuality, it is not too presumptuous to hypothesize that male *G. duebeni* may also decrease sperm to intersex females. In addition to the decreased allocation of sperm, Yang et al. (2008), revealed intersex males actually possess ~15% less sperm than normal males. Other intersex populations as revealed by Ford et al., (2006) also showed a high prevalence of intersexuality in polluted habitats, as well as, a prevalence of parasitic infestations with parasites (i.e. microsporidia). Intersexuality and pollution have not been proven, however there is a strong correlation that might be due to increased host susceptibility. Susceptibility is a complex proposal, but it may be rooted in a compromised immune system. All of the aforementioned costs of intersexuality have been observed and documented in the various populations of amphipods.

The cause and mechanism of intersexuality is not completely understood. Studies and experiments presented have shown that costs are high with intersexuality resulting in reduced reproductive output as compared with normal individuals. Regardless of costs to reproductive output, intersexuality is present and persistent in natural populations (Ford et al., 2008; Glazier et al., 2012). Presence of intersexes in natural populations and the success of these populations imply there are a mixture of factors and complexities not totally known or understood. A better understanding of genetic processes would greatly facilitate the understanding of sex determination and

differentiations while possibly providing insights to the high incidence of intersexuality in FBC and FBR *G. minus* populations.

1.3 Reproduction in crustaceans

Crustaceans consist of a large portion (~50,000 species) of Arthropoda; this group of organisms demonstrates high sexual plasticity, which not only makes sex determination problematic but also quite interesting. Sex determination in crustaceans is known to be determined and/or influenced by genetic factors (Legrand & Legrand, 1987), environmental flux (Bulneim, 1978), and parasitic stressors (Mautner et al., 2007).

There has been and still exists much confusion regarding the definition between intersex and other phenotypic conditions, as well as, reproductive strategies. Sexual, asexual and parthenogenic reproduction are a few of the examples that become confused with the term intersexuality. Numerous examples exist within the subphylum Crustacea (Brūnnich 1772) that represents various life histories and reproductive strategies (Legrand and Legrand 1987; Guler 2012). Strategies represented are described in the following sections and include organisms that utilize sexual, asexual and parthenogenic reproduction.

Sexual reproduction requires two separate sexes, a male and a female, to combine their genetic material (gametes) via fertilization of ovum by sperm. The gametes of each sex have undergone meiosis and are at that point haploid sex cells. Haploid sex cells will combine developing into diploid organisms (Klug and Cummings, 2000). Evolution teaches us that from sexual reproduction progeny should exhibit the most desirable traits from each parent, which then allow the offspring to be more successful (Klug and Cummings, 2000). The successes of organisms that utilize sexual reproduction have contributed greatly, and thus is the predominant manner for animals to produce future generations. However, other sexual strategies evolve that propagate a species when epigenetic or genetic factors inhibit sexual reproduction (Hodgson, 1999). The majority of organisms that utilize sexual reproduction are separate sexes referred to as gonochoristic organisms that develop gametes and bring the gametes together (i.e. mixing the genes) to produce a zygote. Aquatic organisms,

such as, crustaceans can employ either sexual or asexual means of reproduction, which are designated into broad and assorted life styles; 1) vegetative and parthenogenesis are asexual and 2) gonochoristic and hermaphroditic are sexual (Baeza et al, 2009).

Asexual reproduction does not involve the fertilization of ova, meaning that the union of the sperm and egg does not occur. Asexual reproduction is accomplished either by vegetative means (normally plants) or by parthenogenesis (normally animals) (Hodges, 1999). Vegetative reproduction is by means of budding, fragmentation, and fission; examples of vegetative methods (non-plants) that are utilized by aquatic invertebrate phyla are Porifera, sponges; Cnideria, corals, sea anemones, and some echinoderms. Porifera and Cnideria utilize budding to produce colonies; echinoderms utilize fragmentation. The offspring of asexual reproduction are almost always genetically identical to the parent, unless a mutation occurs; there is no mixing of genes (ameiotic) (Butlin et al, 1998). Lack of genetic exchange may counter evidence supporting biodiversity, but lack of genetic diversity may not always be unfavorable for some organisms; exact or near exact replication of an individual may have a successful genotype for a specific ecological condition (e.g. corals) (Butlin et al, 1998).

A second type of asexual reproduction is parthenogenesis. Parthenogenesis (virgin birth) is another form of asexual reproduction in which the ova are not fertilized; but develop into adults (Dictionary.com http://dictionary.reference.com/browse/par-thenogenesis, accessed 12/2017). Parthenogenesis is believed to have evolved after the previous forms of asexual reproduction because there are gametes (ova) formed. There are three types of parthenogenesis classified according to the offspring produced; haploid males (arrhenotoky), females (thelytoky), and where either sex is produced (amphitoky). The two reproductive parthenogenic strategies include cyclic (sexual to asexual) and obligate (asexual only).

The nonmarine ostracods (Arthropoda: Crustacea) are very common, widely dispersed, have been studied extensively and make for an excellent model of reproductive evolution and success; ostracods utilize asexual, sexual, and sometimes both forms of reproduction (Chaplin and Ayre, 1989).

Rossie et al (2010) conducted a wide survey of *Limnocythere inopinata*, (nonmarine ostracods) in Italian and Austrian lakes to determine the potential cause(s) of the species transformation from sexual to asexual reproduction. A total of 812 specimens were collected from twenty-one sample sites throughout Europe (Austria and Italy) in 1995 and 1996. All specimens collected were female with low heterozygosity and genetic diversity. Rossie et al (2010) concluded that *L. inopinata* is a product of geographical parthenogenesis (i.e. post-glacial) that occurred and has appeared to have frozen in this asexual state (Rossie et al 2010).

Sex in *Daphnia pulex* (Arthropoda: Crustacea) is environmentally determined and is another prime example of the evolutionary history of obligatory parthenogenesis (thelytoky) and cyclic parthenogenesis reproduction (Innes and Herbert, 1988). *D. pulex* was the first crustacean to have its genome sequenced (12 chromosomes: 30,000 genes), which also revealed many gene duplicates that diverge and take on new roles (Gilbert, 2009). Theoretical studies once thought that the duplicate genes were unusable or neutral, however; this finding submits that they are conserved and replicated for a purpose and are not useless. Since the completion of the sequencing, duplicate genes have been shown to be conserved under specific environmental conditions/constraints (Kondrashov, 2002) (Figure 2).

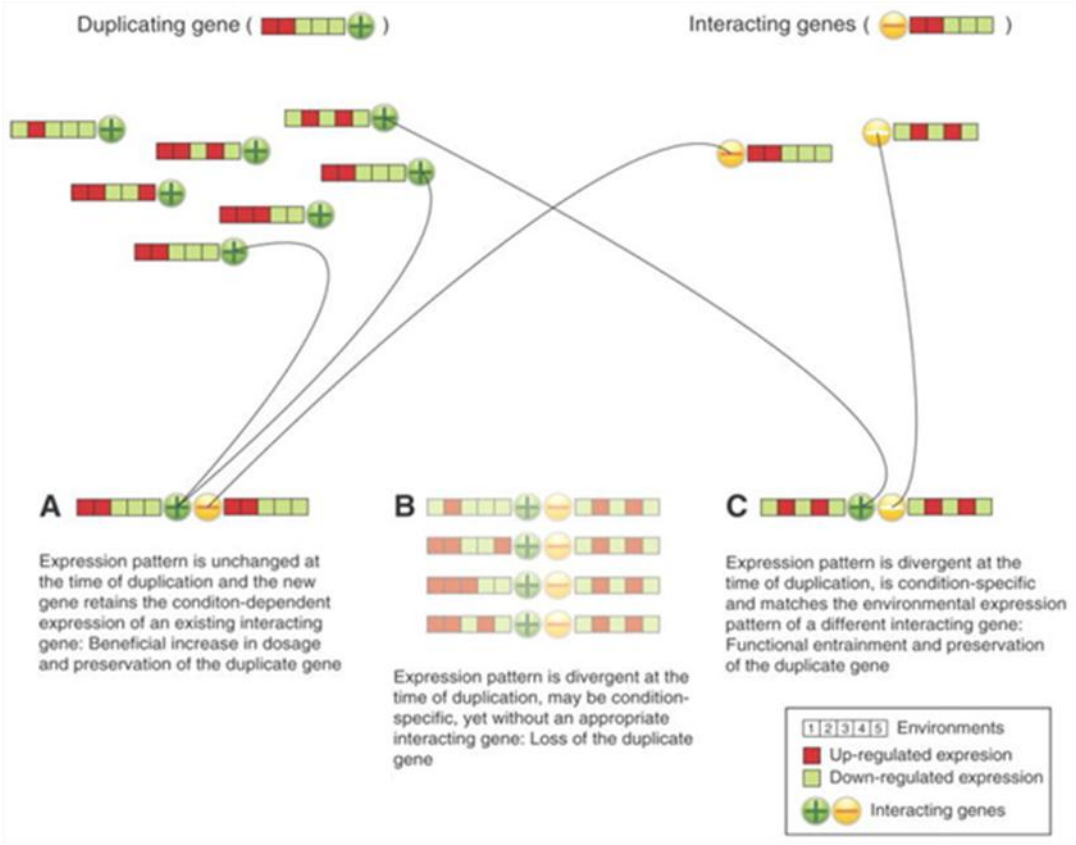

Figure 2. Model of gene duplication under the preservation by entrainment (PBE) model. Figure A illustrates the expression pattern is unchanged at the time of duplication and retains the gene condition and expression. Figure B illustrates expression patterns that are divergent at the time of duplication because they may be condition-specific, the duplicate gene is lost. Figure C illustrates an expression pattern that is divergent at the necessary time of duplication; because it is condition-specific and matches the environmental condition expressed at the time of duplication it is preserved. (Kondrashov et al., 2002).

In general, the *D. pulex* crustacean has the ability to carry many genes packed tightly into its chromosomes, and during the mitotic phase of obligatory and cyclic parthenogenesis retains duplicate genes that are in sync with the environmental conditions. In light of genetic manipulations, this may be something considered in other populations and how anomalies, such as the intersex condition, exists.

Sexually reproducing animals may either be separate sexes (i.e. gonochoristic) or one individual (i.e. hermaphrodites). Most hermaphrodites are solitary invertebrates that have high success and low reproductive costs (Charnov, 1982). Well-known examples of hermaphrodites found in nature (crustaceans, plants and some animals) are often confused with intersexuality. In aquatic organisms there are two kinds of hermaphrodites, simultaneous and synchronous. Simultaneous are defined as a plant or animal having both male and female sex organs or other sexual characteristics, either abnormally or (in the case of some organisms) as the natural condition (Narita et al, 2010). Synchronous hermaphrodites are defined as an adult organism having both male and female sexual organs at the same time, which at one time or another in their life, change sex. Some of the best examples of synchronous hermaphrodites include reef fish, plants and gastropods (Ghiselin, 1969).

Gonochoristic species (dioecism) consist of males and females that are always separate sexes and usually possess secondary sexual characteristics; however, some species like bivalve mollusks (e.g. mussels, clams) require dissection to determine the differences because there are no noticeable secondary sexual characteristics (Baeza et al., 2009). Other gonochoristic species exhibit secondary sexual characteristics (i.e. sexual size dimorphism) that aid in determining males from females, which is believed to be based in sexual selection theory (Baeza et al., 2009).

1.4 Sex determination in Crustaceana

Animal mating systems were first discussed in evolutionary terms by Darwin (Darwin, 1871). Since then, developments in genetic theory and molecular techniques have substantially increased our knowledge of mating systems, sex ratios, sexual dimorphism, and sex determination. To better understand the mating systems of Crustaceana (Amphipoda), we must utilize this knowledge, with regard to the

evolutionary tenets of natural selection (i.e. fitness and intra-sexual competition) (Darwin, 1871). According to Darwin (1871), when one sex becomes a limiting factor for the other, the result is an increase in intra-sexual competition and the cause of sexual selection becomes pivotal to the evolutionary development of a population's sex ratio. In 1958, Fisher put forth the principle of equal investment which ties evolution theory to sex ratios.

The principle of equal investment (Fisher, 1958) is an evolutionary model that explains why sex ratios in gonochoristic organisms is approximately 1:1 (M:F). Fisher's principle is rooted in the concept of frequency-dependent selection as described by Darwin (1871) via natural selection. Fisher's (1958) principle extends the explanation for how natural selection acts on genes and how those genes may cause parents to not equally invest their resources. Overall, sex ratio is the ratio of sexually active adult males available to fertilize sexually active adult females (Correa and Thiel, 2003), which is driven by sex determining mechanisms. At the population level, sex ratios may be skewed or biased, and depending upon the influence may determine the success of the population. Consequently, severely biased populations are at risk of extinction (e.g. strongly male-biased populations) (Martins, et al., 2009). The incidence of intersexuality and sex ratio fluctuation is essential to fully evaluate a population's health (Martins, et al., 2009). Sex ratios in crustacean populations are hardly ever unbiased and in fact, are theoretically expected, as well as commonly observed in animals that are under ESD influence (Charnov, 1982). Sex ratios may also be evidence of fluctuating environmental influences in aquatic ecosystems (Sasher and Qureshi, 2011; Prato et al., 2009; Castiglioni and Buckup, 2008; Ford and Glazier, 2008; Haley, 1985; Hatcher and Dunn, 1998). In some populations, skewed sex ratios could be the result of gender bias in the production of offspring, sexually differentiated mortality (i.e. sex biased predation) (Appadoo and Myers, 2001), differential longevity, and even sex change, as with copepods (Kiorbie, 2006). Sex ratios and proportions of intersexes are related to the classification of true males or females, so if intersexes are inconsistently described as either males or females this may also skew ratios (Gusmao et al., 209). In a copepod (*Paracalanus parvus*), it has been observed that males develop more quickly than females, thus suggesting that stage duration was shorter than that of females (Landry, 1983), thereby the sex ratio would be female-biased. Sex ratios that are in response to an influence (e.g.

cytoplasmic parasites) are known to favour female-biased sex ratios (Kiorbie, 2006). Then theoretically, males in female-biased populations would have less competition, and more opportunity to fertilize females (discussed in Chapter 4).

Seeing that sex determination in amphipods has yet to be clarified and remains difficult to discern, sex chromosomes, ESD's including cytoplasmic parasites, remain popularly studied mechanisms.

1.4.1 Sex chromosomes

The sex chromosome in the majority of arthropods determines an individual's gender and is morphologically distinguishable with high amounts of repetitive DNA (Bull, 1983). The chromosome system is labeled as X and Y (or Z and W) and within the XY system females are the homogametic sex (XX) and males are the heterogametic sex (XY). In the ZW system, males are homogametic (ZZ) and females are heterogametic (ZW) (Legrand et al., 1987). Both systems can be found in Crustacea; Amphipoda, Decapoda and Ostracods all have male heterogametic systems and Branchiopods have female heterogametic systems (Legrand et al., 1987). More karyological studies of Crustacea would be most helpful in discerning sex chromosomes; however, due to the high diploid numbers and small chromosome size, describing structure and function is problematic (Salemaa, 1979). In Gammaridae, the diploid chromosome number (2n) is 52 and the modal haploid number is 26 (see Figure 3). In *G. minus*, the genetic structure is unknown, thus the XY or ZZ systems are presumed based upon the Baikal amphipod karyotype (Figure 3).

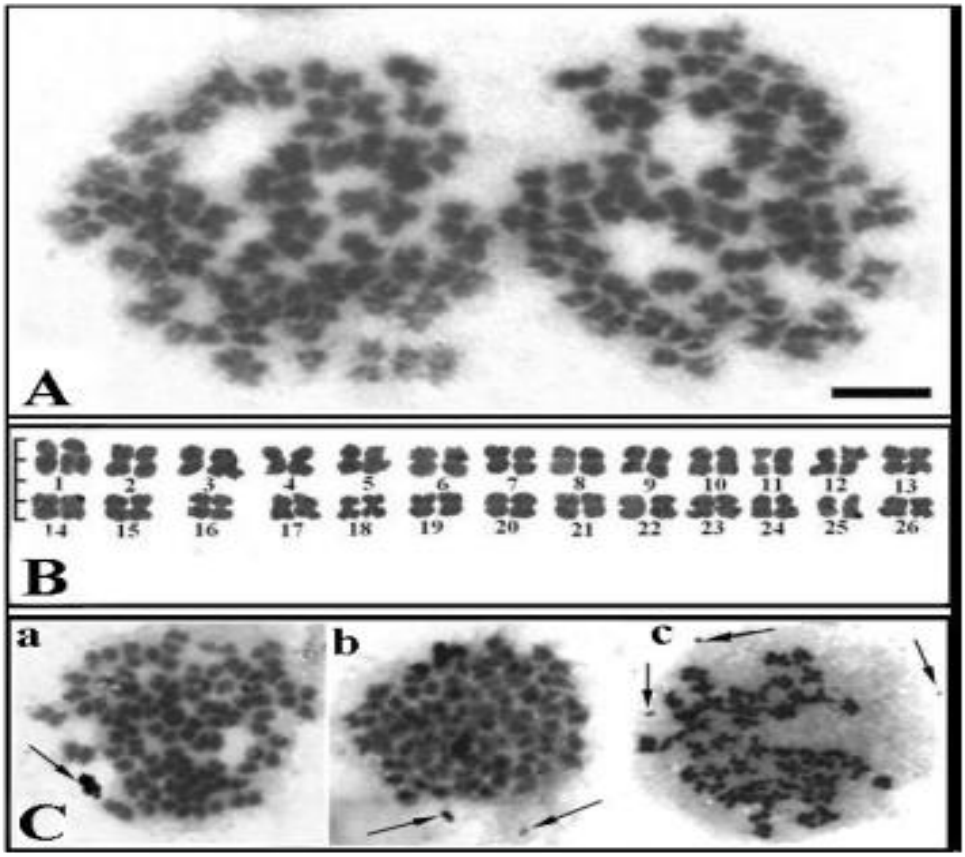

Figure 3: Chromosomes of the endemic Baikal amphipod Polyacanthisca calceolata. (A) metaphase chromosomes of two embryonic cells. Scale bar 10 μm. (B) karyogram of a chromosome set. Scale bar 20 μm. (C) metaphase plates with supernumerary B chromosomes. Scale bar unknown: in the form of normal chromosomes (a), in the form of very small chromosomes (b), and in the form of a few dot heterochromatic bodies (c). Staining with 1% water based fuchsine. Taken from Natyaganova and Sitnikova (2012).

The study conducted by Natyaganova and Sitnikova (2012) on Lake Baikal and seven Gammarus were described with a diploid number of 52 and similar chromosomal morphology. Structurally, chromosomes were consistent (e.g. chromosomal banding, centromere location and arm lengths); similar chromosomal morphology can indicate similarities between species (White, 1973). Specifically, the amphipod *Polyacanthisca calceolate* (Bazikalova, 1937) analyzed by Natyaganova and Sitnikova (2012); revealed a diploid number (*2n*=52) and similar chromosome morphology (i.e. median centromere). Chromosomes varied in size from five to eight µm plus the presence of supernumerary B chromosomes. Genes that are known to be responsible for resistance to unfavorable environmental conditions have been found in B chromosomes and play an adaptive role (Miao et al., 1991).

It is important to mention that some crustacean species are polymorphic and show different chromosomal structural forms (White, 1973), such as, the well studied, *Thais lapillus* (mollusk) located on the Brittany coast. White (1973) provides evidence that chromosome morphs are adapted to different habitats and have a better response to physiological stress (i.e. greater hardiness and salinity tolerance) than diploids. In addition, the freshwater snail *Buliinus truncates/tropicus* complex, occurs naturally in a polyploidy series including diploids (*2n = 2x = 36*), tetraploids (*2n + 4x + 74*) etc. (Goldman et al., 1982). Polyploidy is fairly common in plants and rare in animals (White, 1973), thus to date, polyploidy has not been shown present in Gammaridea (Goldman et al., 1982; Sexton, 1980).

Despite what has been determined due to crustacean karyological studies thus far, much is still unknown. Due to technical difficulties, as previously mentioned, sex chromosomes are not well differentiated in amphipods. Breeding experiments with genetic males and females and neo-males and neo-females may be of help in determining sex chromosome patterns within amphipods.

In 1999, Suzuki conducted cross breeding experiments with the isopod *Armadillidium vulgare* by sex reversal of females. Utilizing three genetic females Suzuki (1999) implanted three ethanol-treated AGs (t-AGs) into each genetic female at various developmental stages. The purpose of ethanol treatment was to stop the production of AGH; leaving the gland with residual hormone. The end point of the experiment was

if the testes developed it was indication of sex reversal within the genetic female. Interestingly, sex reversal only occurred after the female had already become sexually differentiated and not while undifferentiated, which confirmed that AGH is not a sex-determining mechanism, but sex-reversing.

1.4.2 Sex hormones

Charniaux-Cotton (1954) was the first to discover the fundamental mechanism, which regulates sex differentiation, is under hormonal control via the androgenic gland (AG) in Crustacea. Without the development of the AG there will be no hormone secreted to ensure male development and the organism will be female, by default (Charniaux-Cotton, 1954). The AG hormone controls development of the primary and secondary sex characteristics, such as, spermatogenesis and papillae, respectively. Charniaux-Cotton (1965 and 1962) and Khalaila et al., (2001) conducted a series of implantation experiments to determine the effect of the androgenic gland. Utilizing the amphipod *Orchestia gammarellus,* Charniaux-Cotton (1954) implanted females (one juvenile and one matured) with an AG that resulted in the juvenile female reverting to a male and the matured female development of primary and secondary sex characteristics (masculinization). Among the matured females there were several results where the ovary "transitioned" to functional testis producing spermatozoa, spermatids and spermatocytes (Charniaux-Cotton, 1962). Upon the removal of the AG from the male, no further spermatogenesis occurred and the male became undifferentiated per se; also, when an ovary was implanted into males without AG's there was no feminization results, but if the male retained the AG the implanted ovary would be transformed into a testis (Charniaux-Cotton, 1962). The role of the AG has been demonstrated in many other crustacean species, specifically in the red claw crayfish, *Cherax quadriacarinatus.* Khalaila et al., (2001) implanted hypertrophied AGs into juvenile females, and the results to secondary characteristics were as follows; 91.6% of the 12 surviving AG-implanted females, developed male-like propodi, which included the characteristic red patch of males. Furthermore, female characteristics (i.e. wide abdomen, wide endopod and simple setation) were suppressed and anatomically, gonads were yellowish white in comparison to greenish brown ovaries. Transverse section of the AG-implanted female ovaries revealed that the majority of

oocytes were at the lipid stage and a small number of oocytes at the early yolk stage, normally, females have matured oocytes with a perinuclear zone and yolk globules (vitellogenesis). Other implantation studies with similar results include, Taketomi and Nishikawa (1996) and *Procambarus clarkia* (crayfish) exhibiting inhibited vitellogenesis; Fowler and Leonard (1999) and AG extracted-injected female *Cherax destructor* where gonads were inhibited and vitellogenesis was arrested. These findings are in keeping with Charniaux-Cotton and the series of implantation experiments that the AG is the only source of the hormone and it is ultimately responsible for maleness and differentiation of Crustacea.

1.5 *Gammarus minus*

G. minus SAY (1818), is a freshwater amphipod (Crustacea: Amphipoda) (see Figure 4) that is most notably a gonochoristic and sexually dimorphic crustacean with a relatively small distribution. Populations are found in spring and cave systems predominantly throughout the Appalachian Valley and westward toward the Mississippi River (Holisinger, 1984). Amphipods are important organisms that play a crucial role in ecosystem dynamics, mainly as a food source for upper tropic levels (e.g. salamanders, crayfish, and fish) (Glazier et al., 2012, 2009; Miller 1977, Haley 1985).

G. minus is an omnivorous, benthic species that shreds leaf materials, grazes on algae, organic debris and bacteria. They are found in cave streams, springs, and spring-runs throughout a large part of the Appalachians, Interior Low plateaus, and Ozarks (Holsinger, 1972). The two populations of this study in FBR and FBC are both surface dwellers and do not exhibit any cave adaptations. Surface dwellers reside in the first few centimeters of mud and sediment, on and under rocks, in water cress and other plant roots (Buikema et al., 1980; Miller, 1977; Haley 1985), and cave dwellers in caves with aquatic habitats. This species is well adapted to hardened waters (pH 7.0 – 8.0) with dolomite-outcroppings and limestone bedrock (Holsinger, 1972), which is characteristic of karsts geology. Substrate characteristics have been shown to have heavy influence on the abundance and life history cycles of the *G. spp.* (Minckley and Cole, 1963; Culver 1970a and 1970b; Hughes, 170; Culver 1971; Jenio, 1972; Obrdlik, 1972; Rees, 1972; Wallace et al., 1975). Holsinger and Culver

(1970) described three forms based on morphology and development of sensory characteristics: form I is an extreme cave form with underdeveloped eyes and a longer antenna, form II is an intermediate cave form, and form III, a spring form characterized by well developed eyes and a shorter antenna than form I (see Figure 4). In their natural habitat, breeding cycles are similar with many amphipod species and sexual activity can be partly seasonal and/or continuous throughout the year (Pockl et al., 2003). Amphipods are a fine example of an r-selected species.

Figure 4. FBC Male (points 1 – 2, along red arc = 7.5mm) *G. minus* spring form III (taken by Tamela L. Brown 2017).

G. minus is sexually dimorphic and males grow larger than females. Within amphipod populations males may grow up to 16 mm and females 10 mm. Males possess two genital papillae between the last pair of walking legs, and females possessing four pairs of brood plates (oostegites) in the thoracic region (Ford et al., 2007; Glazier, 2009; Ladewig et al., 2002). In most Gammarus amphipods, the first two pairs of peropods are modified; the variation into claw-like structures (gnathopods) is probably due to evolutionary modification based on function and is relative to body size (Borowsky, 1984). The smaller anterior gnathopods of males are larger than the females' anterior gnathopods; the male will hook his gnathopod onto the females' coaxial segment to aid in mate guarding during pre-copula. Mate-guarding is a reproductive strategy used to ensure proliferation of the males' genes (Borowsky, 1984; Jormalainen, 1998). Larger females may create a problem for males, as they are more difficult to control in stream currents, thus there is a risk of the female breaking free and jeopardizing mating. The larger posterior gnathopods remain free perhaps to aid in control while maneuvering in spring currents and/or during agonistic encounters (Borowsky, 1984). Males possess genital papillae (usually two), while females possess four brood plates (oostegites) that hold eggs within the brood chamber. Matured females will present hair-like structures on the oostegites commonly referred to as brood hairs.

Fecundity strongly correlates with female body length; with larger females producing larger (numbers of embryos) brood sizes (Glazier et al., 2012; Ford et al., 2004; Costa and Costa, 1999). During mate-guarding the male and female go into pre-copula position before egg-laying. The female molts and the eggs drop into the brood pouch from the oviducts and then the male externally fertilizes the eggs. On average, Gammarids populations may produce 4 to 16 eggs per brood (Glazier et al., 2012; Miller, 1977; Mohammadi et al., 2010). The female retains the fertilized eggs in the brood pouch until development and hatching occurs, then offspring are released. Once offspring are released, the juveniles develop through a series of growth stages undergoing molts at each developmental stage; once sexual differentiation classifies adults as male or female, then the mature adults will present with the appropriate secondary sex characteristics, defining the phenotype.

1.5.1 Intersexuality in *G. minus*

G. minus is one of the most studied amphipods in the U.S. and yet there are very few reports of intersexes; in addition, Ford and Glazier (2008) sampled *G. minus* in many springs of Pennsylvania and found only two intersexes. Of particular interest are two *G. minus* populations, in Virginia, USA. Both populations are male-biased with extraordinarily high proportions of intersex females (Buikema et al, 1980; Miller, 1977; Ford and Glazier, 2008; Glazier et al., 2012) this high incidence of intersexes is uncommon and presents a conundrum because intersexes should not be successful, as it defies sexual competitive theory, and according to Ford and Glazier (2008), this phenomenon may have persisted for at least the past 38 years, as well as male-biased sex ratios (Miller, 1977; Buikema, 1980; Ford and Glazier, 2008, Glazier et al., 2012).

The cause of intersexuality in amphipods is not entirely understood; however, research shows determinants such as: environmental determination (ESD), feminizing parasites, environmental pollution, and genetics may play a role. Accordingly, this dissertation was designed around various determinants of the developmental biology of these amphipods to discern their unusual sexual phenotype, sex ratios and natural life cycles.

1.6 Statement of the Aims

The primary aim of this study was to investigate the prevalence, causes and costs of intersexuality in the amphipod *G. minus*. Several subsidiary aims of the study included:

- determine the population dynamics: determined sex ratios of phenotypes (males, females, and intersexes) of both FBC and FBR populations; (Chapter 3)
- determine the difference in the body lengths of males, females, and intersexes of *G. minus*; (Chapter 3)
- determine the reproductive cycles of normal and intersex *G. minus* from different populations; (Chapter 3)
- determine the cause(s) of intersexuality in *G. minus* using histological and molecular (PCR) techniques (Chapter 4)
- investigate surrounding populations of *G. minus* to determine the prevalence of intersexed amphipods (Virginia and Ohio); (Chapter 3)
- determine whether there are any reproductive or behavioral costs to intersexuality in *G. minus* (Chapter 5).

2. Site Description

2.1 Site Description

To assess the population dynamics and ecology of the two populations of *G. minus* a field study was undertaken over a three year period. *G. minus* were collected from September 2010 to September 2013 from the Falling Branch Road study area and comparative study sites.

This study focused on the two primary sites on Falling Branch Road in Christiansburg, Montgomery County, Virginia where the high prevalence of intersex females were found (Miller, 1977; Buikema, 1980; Ford and Glazier, 2008; Glazier et al., 2012). *G. minus* adults was collected from three different springs, which are similar in geology (i.e. Karsts and cave spring runs) to the primary study site. The three sites included one in the State of Virginia (Bradley Run), and two out of the State of Virginia (Maryland and Ohio). The collected populations of *G. minus* were examined to determine the prevalence of the intersexed condition within these surrounding areas (Figure 5).

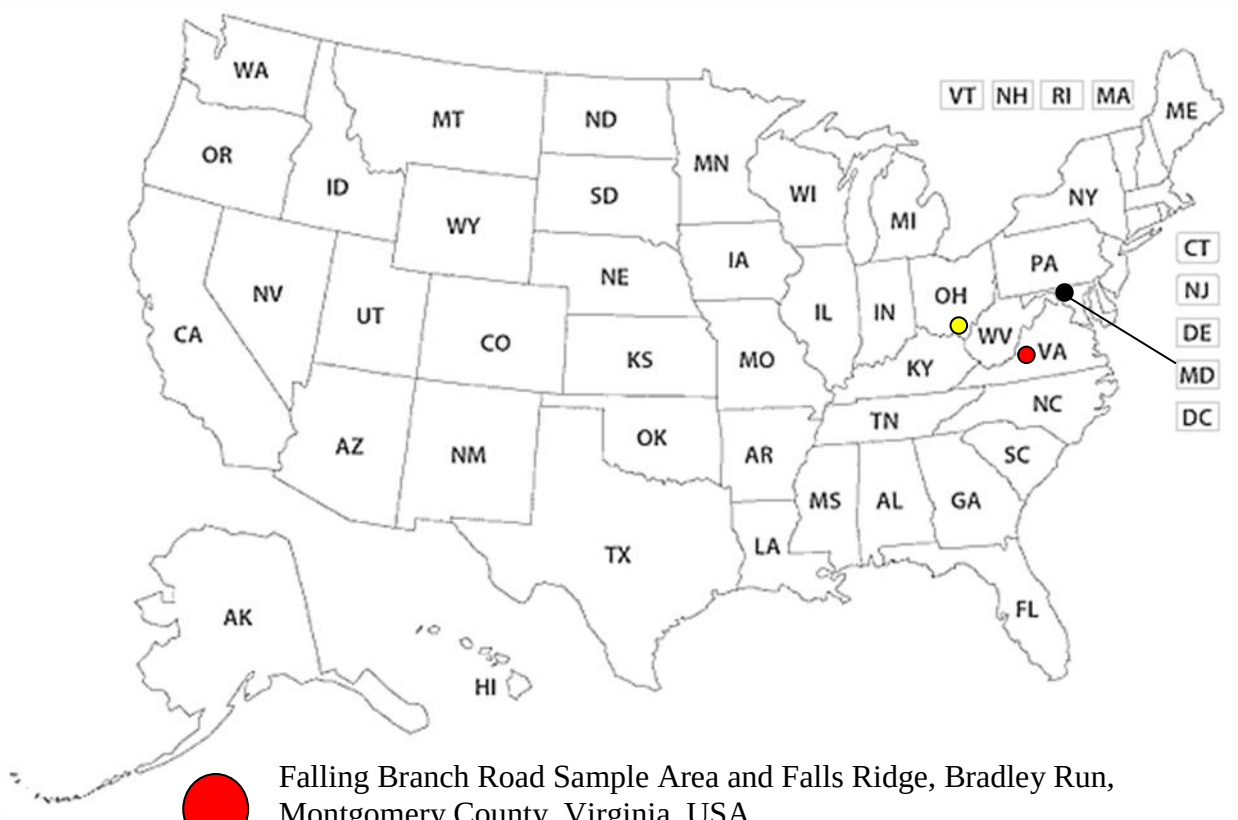

Figure 5. United States Map. Locations of all sample sites where *G. minus* were collected including the comparative sites. The primary sample sites (Falling Branch Road Spring, and Falling Branch Cave Spring, Montgomery County, Virginia) where *G. minus* were collected from Sep 2010 – Sep 2013. Surrounding areas of comparative springs, Bradley Run, Montgomery County, Virginia, Davis Memorial Preserve, Cedar Fork Cave Spring, Adams County, Ohio and Antietam Creek, Washington County, Maryland. Two letter abbreviations represent the individual states. Ohio (OH), Virginia (VA) and Maryland (MD).

The general area of Falling Branch Road is described below.

Falling Branch Road area geological formations date back to the Ordovician period, the second Paleozoic Era, which covers the time between 485.4 and 443.8 million years ago (Department of Conservation, PUB 14, 2018). Montgomery county bedrock is described as thick, gray, coarse-grained, limestone with fine-grained micrite ($CaCO_3$), and dolomite (Department of Conservation and Economic Division of Mineral Resources (DMME). The dolomite is overlain by bryozoan reef complexes with micrite matrix and mudstone (Department of Conservation and Economic Division of Mineral Resources (DMME). The overall thickness of the bedrock is about 122.5 m. The sample sites within this bedrock include FBR, FBC and Bradley Run in Elliston, Virginia. Further description of the primary sample sites follow in separate sections and will include the exact location, geology, and flora and fauna for each site.

2.1.1 Falling Branch Cave and Road Springs

The Falling Branch Cave sample site is a spring – run located within a nature preserve on Falling Branch Road (37.12, -80.33). Falling Branch Road sample site is a spring located approximately 1.9 km from FBC (37.12, -80.35). Both are lotic, freshwater, alkaline, hard water springs found in karsts topography within the Roanoke Virginia Watershed.

Little is known about the streams water quality, other than environmental parameters, which are documented by several studies and this study (see Chapter 3). The Department of Environmental Quality (DEQ), located in Richmond Virginia, monitor downstream of both sites, which also include other effluents from various nearby springs (e.g. Elliot Creek). Unfortunately, the only protocol is for *Escherichia coli* and other coliforms because the streams are remote (DEQ, pers com, 2019). However, in an attempt to further elucidate pollutant sources, florescent stream dye tracing was attempted to locate headwaters and recharging zones of each stream. The headwaters and recharging zones of each stream would help in evaluating surface and subsurface exposures, and if there are pollutant sources (e.g. agriculture run-off,

industry, mining, treatment plants, and other) this would help to determine any potential pollutants. The florescent stream dye tracing was conducted, in conjunction, with The Virginia Department of Parks and Recreation, however it was unsuccessful (data not shown), and both streams (FBC and FBR) water sources, and surface exposures (if any) remain unknown.

Karsts topography is a very characteristic and unique topography, thus karsts warrants a separate descriptive section.

2.1.2 Karsts Topography

In the state of Virginia, the Division of Geology and Mineral Resources (DGMR) identify karsts topography spanning from the western border (with West Virginia) toward the east approximately 2 to 3 counties, and from southern border toward the northern border, inclusive (Figure 6). The guidelines used by DGMR include the following geological features: 1) landscapes are commonly underlain by dolomite, marble and limestone bedrock, 2) landscapes result from the dissolution of bedrock, 3) features include sinkholes, sinking and losing streams, caves, and large springs, 4) extensive underground drainage networks that bypass surface drainage, and 5) the landscape is hosts to many rare organisms, particularly cave specimens (Hubbard, 2011). All of the karsts features may have positive and negative effects on water quality, which in turn, affects aquatic organisms like amphipods (discussed in Chapter 3).

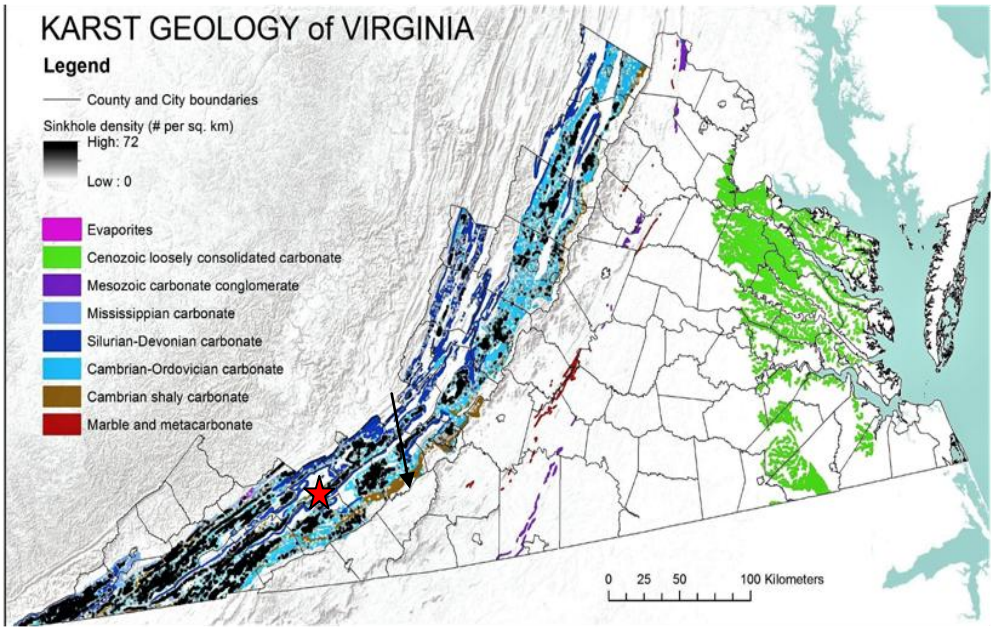

Figure 6. Caves and Karsts of Virginia. The western counties from south to north are characterized as karsts. Montgomery County is identified by the red star. Retrieved from Division of Geology and Mineral Resources (dgmrinfo@dmme.virginia.gov. retrieved 8/25/18).

Karsts landscape is primarily developed because the underlying bedrock (limestone, dolomite or other soluble rock) go through a complex stage of dissolve as water enters the system from precipitation, surface runoff and streams. As groundwater dissolves limestone and/or other soluble bedrock, it creates fractures and voids that potentially cause the land to collapse and create sinkholes (Hubbard, 2011). Sinkholes become part of the recharging zone for the underground water system, which is referred to as the autogenic recharging zone. Autogenic features are part of the natural drainage system that has been created by erosion and includes sink holes, infiltration and soils. Outside of the autogenic recharge zone are sinking streams referred to as allogenic. Allogenic features include sources of recharge that are outside of the naturally eroded zone (Hubbard, 2011). Karsts terrain is very convoluted and dynamic (Figure 7). The discussed features are all within karsts terrain.

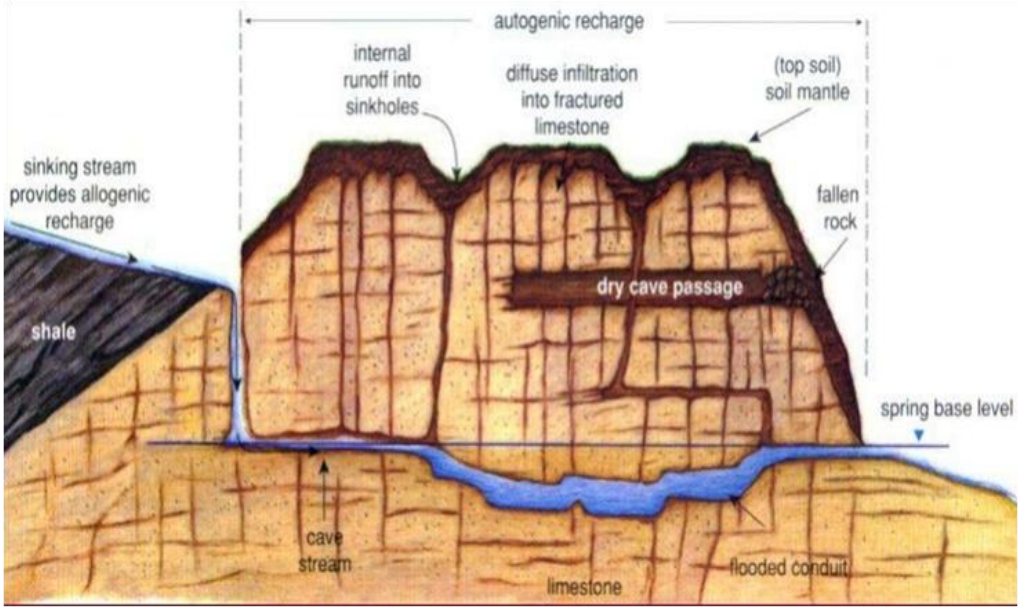

Figure 7. Cross section of karsts terrain. The autogenic recharge area includes, runoff, sink holes and infiltration of water. The allogenic region includes sinking (lost) streams outside of the natural drainage zones. The cave stream depicted is typical (Retrieved from dgmrinfo@dmme.virginia.gov. retrieved 8/25/18).

As water moves through karsts terrain it undergoes specific geochemical reactions. All environmental parameters will be affected, but the primary constituents (temperature, pH, alkalinity and hardness) will determine whether the aquatic environment is habitable and what organisms evolve to survive (Fong et al., 2013).

The geochemical process is described in the following sections, and is based upon the United State Geological Society (USGS).

Natural buffering systems in stream waters (shock absorbers) quickly modify various chemical reactions, so that little or no change results. Chemical changes in aquatic systems can affect bioavailability of nutrients (e.g. phosphorus, nitrogen, and carbon), as well as solubility and toxicity of various chemical constituents (e.g. lead, copper, and cadmium). If environmental change occurs (i.e. pH, temperature, dissolved oxygen) all of which would be altered temporarily or even permanently, if it were not for the natural buffering capacity of aquatic systems.

Water streams from outside (influent) the underground system include precipitation, run off, and surface water bodies. Initially, water is acidic due to atmospheric and soil conditions (rain, pollution, etc.). As water infiltrates through the fractures of the bedrock, it dissolves dolomite, limestone/or other soluble rock, which suspends carbonates in water and is referred to as carbonate alkalinity.

Carbonate alkalinity tends to make up the majority of the total alkalinity, due to common occurrences of dissolution of carbonate rocks in the presence of CO_2. Alkalinity also includes other elements such as, borate, silicate, phosphate, hydroxide and conjugate bases of organic acids. Alkalinity in stream environments is particularly important in determining the ability to neutralize acidic pollution, thus alkalinity is a measure of the buffering power of a stream to minimize pH (H^+ ion concentration) fluctuations. In karsts the buffering compounds (bicarbonates) include the carbonate-bicarbonate system (H_2CO_3, HCO_3, and CO_3). The carbonate-bicarbonate system is basically the absorption of H^+ ions which were released by the geochemical reactions of water, carbon dioxide and soluble bedrock.

The pH, in aquatic systems determines the solubility (what can be dissolved in the water) and bioavailability of nutrients to aquatic life. During photosynthesis the vegetation uses up dissolved carbon dioxide (CO_2), which increases the acidity, while decomposition of vegetation releases CO_2 and dissolves as carbonic acid ($CaHCO_3$) lowering the pH. Subsequently, because the water is acidic, $CaHCO_3$ dissolves calcium carbonate rock, which will release carbonates and neutralize the soil and stream water; as a result, $CaHCO_3$ content, largely determines the pH of the ecosystem. Another parameter that is measured to characterize a stream is hardness. Simply defined, hardness is the amount of metal ions (calcium and magnesium) in water (the higher the hardness, the more dissolved minerals, both calcium and magnesium). Alkalinity and hardness measurements are normally similar because calcium, magnesium, bicarbonate, and carbonate ions in water are derived from limestone solution in geological deposits. It is this geochemical process that creates the alkaline hard waters and other features normally found in karsts areas.

The fauna of karsts (particularly caves) contains unusual organisms that are specialized to their habitats. For example, some organisms only live in caves while others remain in the small cracks and crevices. Organisms in these environments evolve unique adaptations that assist in their survival, such as sensory organs (i.e. long antennas that can detect mates in darkness) (Culver et al., 1995).

In the study sites of FBC and FBR (Figures 8, 9 and 10), when acidic water dissolves the limestone/dolomite found in this area, bicarbonates are formed that raise and stabilize pH and hardness, which results in alkaline, hard water springs (USGS PUB 14, 2018). The organisms (i.e. *G. minus*) that survive there are adapted to all of these environmental parameters (i.e. pH, alkaline, hardness and temperatures).

Figure 8. Location of both sample sites within Montgomery County, Christiansburg, Virginia, USA, where *G. minus* amphipods were collected from 2010 to 2013. Sites are shown together and the distance from Falling Branch Road (1) sample site to Falling Branch Cave (2) sample site and is approximately 1.9 km (Scribble Maps, May 2018).

Figure 9. Falling Branch Road *G. minus* sample site 1. Elevation: 540 m above mean sea level (37.12, -80.34). Sampling area varied from 0.5 – 1 m in width and 1 – 10 m in length. (Scribble Maps, May 2019).

Figure 10. Falling Branch Cave *G. minus* sample site 2. Elevation: 619m above mean sea level (37.1286, 80.3302). Sampling area varied from 0.5 m – 1 m in width and 1 – 5 m in length. (Scribble Maps, 2019).

2.2 Falling Branch Cave

This spring emerges from a small cave found at the base of the mountainside and flows through a meadow towards the Falling Branch Tributary and onto the Roanoke River, all of which is a part of the Roanoke River Watershed, Montgomery County, Virginia. The elevation is 543 m above mean sea level (msl) and the topography is defined as karsts. The water source is not readily observable as the spring emerges from a small cave that is impassable, but the spring-run extends approximately 30 m until it joins the Falling Branch Tributary. The sample site where specimens were collected ranged from 8 to 10 m from the mouth of the cave; in this area water depth varies from 0.05 to 0.1 m and the stream width from 1 to 2 m wide. There are two habitats within the stream bed available to the amphipods, water cress (*Nasturtium officiniale*) and bare gravel. Each of the stream bed habitats provide different resources for the amphipods, the water cress provides protection and food for larger amphipods, while bare gravel provides protection for smaller adults and juveniles within the small interstitial spaces (Miller, 1977; Haley, 1997). The gravel substrate varied from < 0.05 – 13.3 mm in size (Miller, 1977). The environmental parameters spring temperatures (^{o}C), dissolved oxygen (mg/L), pH, alkalinity and hardness ($CaCO_3$ mg/L) are detailed in chapter 3.

2.3 Falling Branch Road

The FBR spring emerges from a small opening located on the side of the mountain The spring flows along a deciduous forest (Maples, Popular, and Ash) through various landowner properties, pipes and conduits, as it subsides and re-emerges, until finally converging with Elliot Creek in 6.1 km. The elevation is 591 m above msl and the terrain is conducive to surface runoff entering the stream. The area sampled was a shaded riffle approximately 10 m in length, 1.0 – 2.0 m wide and 1.12 km from the earliest appearance. Water depth fluctuated as precipitation and run off events occurred (per obs) from 0.05 – 0.1 m. Substrate available to aquatic fauna was aggregate rock and smaller sediment (silt-like) free from aquatic vegetation. The FBR spring is not nearly as robust at the source as FBC (per obs). The environmental parameters spring temperatures (^{o}C), dissolved oxygen (mg/L), pH, alkalinity and hardness ($CaCO_3$ mg/L) are detailed in chapter 3.

Both FBC and FBR were the primary sample sites for this study. The population data was compared and contrasted with *G. minus* from the other like sample sites: Bradley Run, Falls Ridge Preserve, Virginia; Cedar Fork Cave, Davis Memorial Preserve, Ohio; and Antietam Creek, Washington County, Maryland USA.

2.4 Falls Ridge, Bradley Run

The Bradley Run sample site is located in the Falls Ridge Preserve of the Nature Conservancy (in Montgomery County Virginia) and is approximately 6 km north of Falling Branch Road. Bradley Run was studied by Carol Haley (1997) therefore; the stream characterization is based upon that study. The stream is lotic, hard water, alkaline tributary, of the North Fork of Roanoke River, and is a spring-fed, first-order stream at an elevation of 515 m above msl. Furthermore, the stream morphology includes steep riffles alternating with pools and steep banks as the stream meanders through the preserve. The banks are lined with shrubs and deciduous trees, but there is no aquatic vegetation at the site. The study site was approximately 3 meters long and 3 to 5 meters wide with the water depth approximately 5 – 15 cm. As reported in Haley (1997), a striking feature of Bradley Run is the heavy encrustation of marl that covers the rims of pools and any material, including leaves that fall into the stream. Marl is a calcium carbonate or lime-rich mud created in high pH environments (Cole 1983). Water chemistry and temperature for Bradley Run was recorded by Haley (1997) and is summarized as follows: stream temperature ^{o}C (8.0 – 16.6), pH averaged 9.5. The remaining water chemistry (alkalinity, hardness and dissolved oxygen) is comparable to FBC (detailed in Chapter 3).

The population of *G. minus* found in Bradley Run was collected (Figure 11) to use as a comparison of phenotype with *G. minus* in the FBC and FBR sites, because, Carol Haley (1997) had investigated parameters of the sample site, morphology and life cycles of the amphipods, this was not repeated. *G. minus* were only examined for the intersex condition.

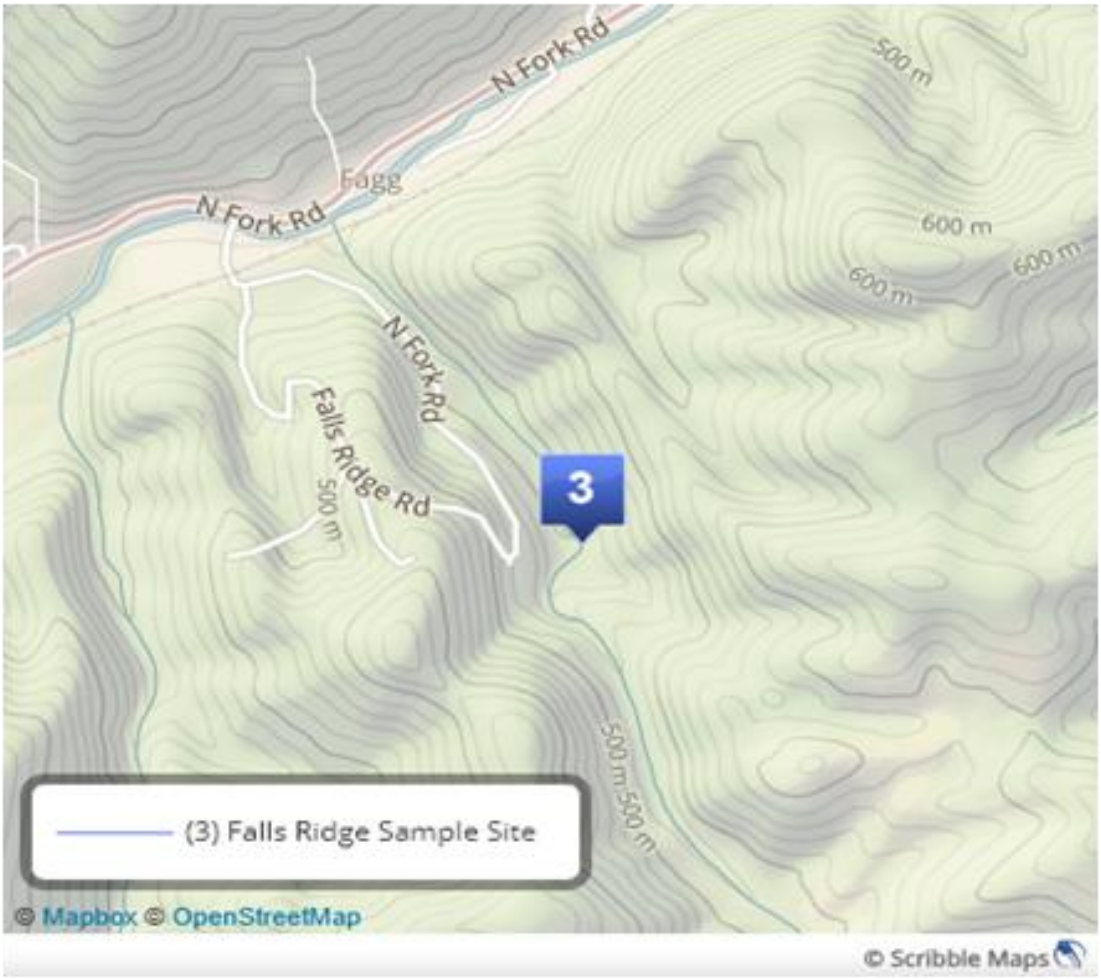

Figure 11. Falls Ridge Conservancy (Bradley Run). *G. minus* (n = 106) were collected from the site as part of a comparative study of amphipods in the surrounding area of Falling Branch sample sites. The site is located northeast of Falling Branch Road sites within the Falls Ridge Conservancy in Montgomery County Virginia (37.1923, 80.3227). (Scribblemaps.com February, 2019).

Outside the State of Virginia, sample sites included Cedar Fork Cave Spring (Figures 12 and 13), Adams County, Ohio and Antietam Creek Spring, Maryland (Figure 14).

2.5 Cedar Fork Cave

Cedar Fork Cave Spring, located in the Davis Memorial Preserve in Adams County, Ohio was the first site and one of only two locations where, *G. minus* are found in Ohio (Beckett et al. 1977). The sample site dropped steeply in elevation from the cave mouth and dumped into a rather large pool and stream (Figure 12 and 13). The site was surrounded by deciduous forests and dolomite outcroppings. The Ohio History Central Organization (www.ohiohistorycentral.org/w/ Bedrock Geology of_Ohio, retrieved 8/2018), cites the origin and the following description of bedrock found in this area. The bedrock dates back to the Ordovician (485 – 444 mya) and Silurian Periods (444 – 416 mya), which is described as thin beds of limestone that alternate with exposed Ohio shale. During the Silurian Period, Ohio was in the tropical latitudes and near the equator. The shallow sea that flooded the state was dominated by limestone, dolomite, gypsum, anhydrite and halite precipitates. Both Ordovician and Silurian Periods, are of marine origin with extensive limestone fossils (Ohio Division of Geological Survey, 2006, retrieved 8/2018). The bedrock in this area gives rise to alkaline, hard waters that are similar in characteristics to FBC and FBR.

The spring-run that emerges from the cave flows 35 m downhill to a pool of water that eventually converges with Cedar Fork Creek, Adams County, Ohio. The streambed is composed of an aggregate of small to medium sized pebbles and rocks (0.5 – 15 cm), with fine silt-sand underneath and no aquatic vegetation. Stream temperature (15^{o}C) and pH (7.8) measurements are the only environmental parameters available for this study. The banks of the spring and pool are lined with various shrubs and trees, including threatened and endangered species. In the prairie opening, American aloe, dwarf hawthorn, hairy wing-stem, purple coneflowers and limestone Adder's tongue fern are present (The Nature Conservancy, www.nature. org/ ourinitiatives /regions/ northamerica/unitedstates/ohio retrieved 8/2018). As with the Bradley Run sample site, the population of *G. minus* found in this spring was collected to compare (intersexes) with the *G. minus* of FBC and FBR.

Figure 12. Cedar Fork Cave. Located in Davis Memorial Park Adams County, Ohio (240 msl, at 38.9270, 83.3741), where *G. minus* were collected.

Figure 13. One of two locations where *G. minus* (spring form) is found in the State of Ohio. Cedar Fork Cave Spring, Adams County, Ohio where the stream emerges from the cave and drops in elevation. (Taken August 2014 by Tamela Brown).

The final site (Antietam Creek) in Washington County, Maryland USA has been studied by Dr. Daniel Fong, American University, Washington D.C., therefore samples of *G. minus* were kindly provided by Dr. Fong. The following section describes the geological formation of the area according to the Maryland Department of Natural Resources (MDNR/gov, retrieved August 2018).

2.6 Antietam Creek

The *G. minus* amphipods from Antietam Creek Spring, Maryland USA (Figure 14) was kindly provided by Dr. Daniel Fong of American University. The Antietam Creek sample site is a located in Washington County, Maryland USA and is characteristic of karsts topography (Fong, per comm., 2018). Washington County is in the western section of the state and lies in the Valley and Ridge Province of the Appalachian Mountains. The site is a small karsts spring found at the base of a cliff which rises 3 m. The spring-run flows 20 m prior to converging with Antietam Creek (Fong, per comm., 2018) and is commonly referred to as the valley of carbonates (Maryland Department of Natural Resources and Maryland Geological Survey). Bedrock in this region consists of inter-bedded and cyclic limestone and dolomite that is 760 m thick (Brezinski, 2013). The lower 180 m of limestone bedrock is thicker than the dolomite and progressively changes in the eastern and far western sections of the Valley and Ridge Province until the dolomite becomes thicker than limestone (Brezinski, 2009). Throughout Washington County are many sinkholes, caves, spring-runs and disappearing streams as is indicative of karsts topography.

Figure 14. Antietam Creek Spring, Maryland (39.4173 -77.7455). Adult *G. minus* specimens were provided via Dr. Daniel Fong from The American University, in December 2012. (ScribbleMaps.com 2018).

The karsts terrain, alkaline hard waters and *G. minus*, were the common thread for all of the study sites.

The following chapter (Prevalence of Intersexuality in *G. minus*) discusses the environmental parameters and population structure for both FBC and FBR, as well as the comparative sites.

3. The prevalence of intersexuality in *G. minus*

3.1 Introduction

The phenomenon of intersex, defined as having both male and female secondary characteristics, exist throughout the animal kingdom (Reinboth, 1975). The cause of intersexuality is not well understood, however, the condition is well established within crustaceans and widespread throughout many amphipod populations, to that end, there is a large body of evidence that consists of publications, preserved specimens, and records that corroborate this phenomena (Sars, 1895; E. O. Sexton, 1906 and 1924; Tattersall, 1910; Sexton and Huxley, 1921; Buikemia, 1930; Buikemia and Miller, 1977; Dunn et al, 1990; Landewig et al., 2003; Ford and Fernandes 2005; Ford and Glazier, 2008; and Glazier, et al., 2012). Records of intersex amphipods exist from as early as the late 1800s' and the occurrence varies widely amongst populations of different species (Ford and Fernandes 2005). Subsequent to this fact, is a discussion of various publications, among amphipod intersexes, which are found in different species and populations from the late 19th Century and onward (Sexton, 1906 and 1924; Tattersall, 1910; Sexton and Huxley, 1921; Dunn et al, 1990; and Ford and Glazier, 2008). In 1895, *Tmetonyx similis* (Sars) was the first recorded intersexed amphipod species (Sexton 1906). The large ovigerous female was collected from the Bay of Biscay; the female measuring 18 mm had developed the secondary sexual characteristics of the male rostrum (Sexton 1906). Sexton and Huxley (1924) gave a very detailed account of male and female intersexes in a population of *G. chevreuxi*, a brackish-water amphipod. *G. chevreuxi* was held in laboratory stock for genetic studies, which was where the intersexes were first noted; but, in February of 1922 intersexes of the wild type of *G. chevreuxi* were captured in the ditches of a salt marsh in Chelson Meadow, Plymouth, U.K.

Other literature that documents the prevalence of intersexed amphipods was investigated by Ford and Fernandes (2005). They reviewed relevant literature along with preserved museum specimens, with the objective of determining how widespread intersexuality occurs amongst amphipods. The literature review supported the records of intersexed amphipods back to the late 19th Centrury (Sars, 1895; Sexton & Huxley,

1921) and forward. Additionally, it was noted that intersexed species varied, as well as, both single and dual-gender intersexes were reported; for example, in some species only female (*G. duebeni,* Dunn et al., 1990; *G. fossarum,* Ladewig et al., 2002), or only male (*Corophium volutator,* Barbeau & Grecian, 2003) phenotypes had been reported (as cited in Ford and Fernandes 2005). The two major outcomes from their survey included; one, evidence that intersexes do occur in a variety of habitats and geographical locations and two, frequency per population is generally relatively low. Bulnheim (1975) sampled three different populations of amphipods that resulted in the prevelance of intersexes as follows; *G. duebeni* (0.7%), *G. salinus* (3.4%), and *G. locusta* (0.8%). Intersexes in *G. minus* are supported by several studies as occurring in low frequency or not at all, however initially recorded in 1977 were two populations with 100% of females displaying the intersexed condition.

Miller (1977) was the first to evaluate the life history and abundance of two populations of the spring form of *G. minus* from Virginia (USA) and the factors that influence the two separate populations. During Miller's (1977) investigation a total of 12,000+ organisms were collected and it was documented that all matured female animals (ovigerous and nonovigerous) were atypical. Each matured female possessed papillae, as well as the normal female secondary sex characteristic. Miller (1977) along with, Buikema (1980), Haley (1997) and Ford and Glazier (2008), have each sampled and observed the populations intersex condition in Falling Branch Cave and Falling Branch Road, as well as noting male biased sex ratios.

During the collections in FBC, Buikema (1980) noted that intersex females consisted of 60% of the total female population, not the 100% that had been previously reported in 1977 (Miller, 1977). In an effort to explain the occurrence of female intersexes in Falling Branch Cave spring, Buikema et al. (1980) put forth two hypotheses; one, the feminizing microsporidian *Octosporea effeminans* was present in the female reproductive system and two, *G. minus* evolves from a functional female into a functional male with age. The 68 animals that Buikema et al. (1980) collected were subsequently characterized into eight categories (Table 1). Three characteristics used to describe females were whether or not they carried a brood, if they possessed papillae, and their size; males were also characterized by their size and if they were intersexed (possessed brood plates).

Table 1. FBC samples examined by Buikema et al (1980). *G. minus* classified into 8 descriptions; number (n), amplexed, sex (M, F), phenotype (Intersexed, Normal), female condition (ovigerous or nonovigerous), and number per size class (mm).

Category	***(n)***	
Amplexed (pairs)	7	
> 7mm length	9	
Total collected	68	
	Males (*n*)	Females (*n*)
Total collected	26	35
Normal	26	5
Intersexed	0	12
Ovigerous females		17
Nonovigerous females		18

A second detailed study, of the FBC population was completed by Haley (1997), which compared and contrasted *G. minus* populations collected from FBC and Bradley Run, both within Montgomery County, Virginia, USA (1997).

Haley (1997), looked at two separate populations of *G. minus* to compare life cycles, secondary production, and correlated habitat characteristics (food source) with the differences observed. In the Haley (1997) study, the population referred to as Richards Creek is the same stream and population as FBC. Richards Creek and Bradley Run amphipods have two different life histories and breeding cycles. Bradley Run breeding cycle is annual; accordingly the amphipods are mature by August and enter the breeding cycle from the fall to winter seasons (September to March), Richards Creek *G. minus* populations breed year around as immature, mature, and ovigerous females were always present. Both populations life histories (Bradley Run and Richards Creek), appear to be related to habitat, the habitat conditions in Bradley Run fluctuated in temperature, pH, and available food sources, while Richards Creek is the more stable environment (temperature, pH, and food sources). Habitat also appears to have an influence on annual productivity. In Bradley Run productivity was 1.8 g/m^2 and in Richards Creek 3.9 g/m^2. Population density was higher at Richards Creek (the more stable environment) where more diverse food sources exist that may be attainable to all stages of the amphipods. For example, algae, biofilms, aquatic macrophytes, and smaller substrate would appear to support immature amphipods; in contrast, Bradley Run's substrate is not as plentiful in algae, aquatic macrophytes, or other food sources. The next section describes the results of the Haley (1997) study regarding amphipod mouthparts and habitat of the two populations.

Nine mouthpart and foregut structures were compared between the two populations and between immature and mature amphipods, as well as feeding behaviors. Mouthpart size was correlated with body size of the respective amphipod. Of the correlations the increase in setae numbers on maxilliped and the ampullae hook were greater for immature than mature individuals. In Richards Creek amphipods, the number of hook setae was greater than in the Bradley Run population. The following mouthparts and foregut were correlated; the outer plate of the maxilliped and the number of cuspidate setae, length of dactyl on the maxilliped palp, and the number of

hook setae on the foregut ampullae. ANCOVA confirmed a statistically significant difference between the regressions of numbers of ampullae setae/body length, of both populations ($p = 0.0001$).

When animals were presented with ground-up leaf material in the laboratory, twenty-one behaviors, grouped into six broad categories, were observed. The frequency of the six behaviors (eating, swimming, gathering, searching, pulling appendages through gnathopods, and handling fecal pellet) was found to be statistically different between immature and mature animals. Both populations engaged in all six categories of feeding behaviors, and in general, mature amphipods gather food with their gnathopods, as well as grooming their antennae, while immature amphipods spent more time searching and discarding food.

Lastly, breeding cycles and ovigerous females were recorded. Based on the presence or absence of ovigerous females, it was determined that in Richards Creek *G. minus* breed all the year around, while the *G. minus* in Bradley Run maintain a seasonal cycle.

Important conclusions made by Haley (1997) included: 1) the two populations differed in features of life history and secondary production, 2) there was some differences in mouth parts and foregut morphology, 3) the immature and mature *G. minus* differed in frequency of feeding behavior and mouthpart morphology, 4) there is a relationship between habitat organic matter and these differences. Overall, the study of these freshwater gammarids provide evidence that stream habitats with finer substrate, supported higher levels of production, than did stream habitats with coarse substrates (Haley, 1997).

Haley (1997) also noted a large number of intersex females at Richards Creek, but not Bradley Run (per comm., 2013) unfortunately the number of females displaying the intersex condition was not quantified.

Ford and Glazier (2008), set out to confirm the high prevalence of intersexes in the Falling Branch Road Springs and compare with other surface and cave springs within the Appalachian areas of Pennsylvania, West Virginia and Virginia (USA). To

properly compare the population of FBC (spring emerges from a small cave) troglobitic forms from Organ Cave system in West Virginia (USA) were also collected. Approximately 200 – 300 amphipods were collected from each site utilizing the standard kick – net sampling protocol, as well as utilizing small hand nets (1 – 1.5mm mesh size) for a total of 2843 specimens. Histology was conducted searching for feminizing parasites, which was completed on sub-samples (x20) of FBC males, normal females and intersex females. Quantification of ovigerous females (normal versus intersex) was reported, as well as enumeration of embryos and their developmental stages (Ford and Glazier, 2008). The results from this important study are summarized below and in Table 2.

Table 2. *G. minus* males, normal females and male and female intersexes from springs located throughout the Appalachian Mountain states of Pennsylvania, West Virginia, and Virginia, USA. Numbers of organisms (n), intersex organisms are expressed in per cent (%) of total female populations. Table is redrawn from Ford and Glazier (2008).

Spring Location	**Year**	**Males *(n)***	**Females *(n)***	**Intersex Males *(n)***	**Intersex Females *(n)***	**Intersex Males (%)**	**Intersex Females (%)**	**Total *(n)***
Petersburg Spring, Pennsylvania, USA	2005	112	133	0	0	0	0	255
Warm Spring, Pennsylvania, USA	–	73	50	0	0	0	0	123
Blue Spring, Pennsylvania, USA	–	107	70	0	0	0	0	177
Arch Spring, Pennsylvania, USA	–	166	161	0	0	0	0	327
Emma Spring, Pennsylvania, USA	–	182	116	1	1	0.5	0.9	300
Linden Hall Spring, Pennsylvania, USA	–	87	123	0	0	0	0	210
Organ Cave, West Virginia (Stream 1), USA	–	197	130	0	0	0	0	327
Organ Cave, West Virginia (Stream 2), USA	–	52	34	0	0	0	0	86
Organ Cave Resurgence, West Virginia, USA	–	104	98	0	0	0	100	202
Falling Branch Cave Spring, Virginia, USA	2006–	211	37	0	109	0	89	357
Falling Branch Cave Spring, Virginia, USA		177	33	2	89	0.7	100	301
Falling Branch Road Spring, Virginia, USA	–	100	0	2	76	2	100	178

Of the total 2843 amphipod specimens collected and examined for intersexuality, 1392 were within the state of Pennsylvania (outside of FBC and FBR), there were 2 (1 male and 1 female) and both were found in Emma Spring in Pennsylvania (USA). Three hundred and fifty-seven animals were observed in 2005 from FBC where 109 were intersex females, thus, representing 74.7% of the total female population. In 2006, 301 specimens were observed from FBC where 89 females and 1 male that presented intersexuality were found, which accounted for 73% of total females and 30% of the total population. All of the females observed and 2 males at FBR were intersexed (78/178). In both, FBC and FBR, the intersex subpopulations were statistically significant from all other streams in this study (Ford and Glazier, 2008). Other differences noted but not statistically significant included, intersex specimens had reduced fecundity when compared with normal females, there were no feminizing parasites found, and frequency of intersexes was very low in the other springs investigated. The overall summation was that Falling Branch Cave and Road Springs both, since first discovered in 1977 remain remarkably populated with intersexed females and male biased sex ratios (Ford and Glazier, 2008).

Field data collected and put forward in this chapter will provide a long term (3+ years) baseline of information regarding the two highly, and persistently intersexed populations of Falling Branch Cave and Falling Branch Road springs.

3.2 Aims and Objectives

The overall aim of the work reported in chapter 3 was to investigate population dynamics of *G. minus* within the two springs under study and compare and contrast these findings with other known amphipod (*G. minus*) populations within the same geographical area. The following objectives ensured that the overall aim was successfully completed.

To monitor the population dynamics of *G. minus* from Falling Branch Cave Spring and Falling Branch Road Spring (Virginia, USA) over three years and record: sexual phenotypes, sex ratios, amphipod lengths, and maturation.

To record and determine the relationships between environmental parameters (stream temperature, pH, dissolved oxygen, alkalinity and hardness) and population variables in *G. minus* from Falling Branch Cave spring and Falling Branch Road spring.

To conduct in-depth analysis of environmental parameters specific to FBC and the decreased sex ratio for this study between the two sample sites (FBC and FBR) and trends or changes over time.

Conduct comparative analysis of sex ratios for the two populations of *G. minus* located in Falling Branch Cave and Road springs with other populations of *G. minus* found in the studied sites listed in Table 1, and subsequent sites sampled in this study (Ohio, Virginia, and Maryland).

3.3 Materials and Methods

3.3.1 Field collections from Falling Branch Cave and Road Springs

To assess sex ratios and determine the prevalence of intersexuality in *G. minus*, specimens from the two spring runs (FBC and FBR) were collected bimonthly from September 2010 to September 2013. A total of 2961 animals were collected from FBC (1613) and FBR (1348). Both sample sites were located in Montgomery County, Virginia approximately 1.9 km apart.

During each sampling event 100 – 140 adult animals (≥5mm) were collected from each habitat, preserved on site and examined in the laboratory; sexual differentiation was an important constituent of the study so only adult and sexually matured specimens were required. *G. minus* is usually sexually matured at a body length ≥ 5 mm, when measured from base of the first antenna to the base of the telson, and can be classified as male or female by the development of secondary sexual characteristics (Bulnheim 1975).

3.3.2 Field Studies: Sex Ratios, amphipod length, and maturation.

Specimens were collected utilizing two methods; one, the standard kick-net method and two, by hand. The standard kick-net technique was conducted as follows: a small hand-held aquarium net (3 mm mesh) was positioned in the water at arms' length downstream and, the substrate was disturbed by foot upstream so that organisms were dislodged and carried into the net. The kick-net method continued from 60 to 120 minutes for both sites. The contents of the net were then emptied into an enamel pan that contained stream water. The second method of collection included hand-picking animals from around rocks. Spring water containing the amphipods was then filtered through a - 4mm mesh sieve (US No. 4) to visually separate out the adults. Next and prior to preserving the animals, they were anesthetized using spring water (1 liter) and one carbon dioxide pellet. Several preliminary tests had been completed to determine the amount of spring water and CO_2 pellets required. CHENGYU Aquarium-CO_2 (ASIN# B07PJH15DJ) pellets were allowed to dissolve and amphipods' were then submerged approximately five minutes prior to transferring into 95% ethyl alcohol (Nilson et al. 2006).

At the laboratory, all live specimens were immediately transferred to microcosms that had been acclimated for one week and held in the environmental chamber; specifically, the containers were prepared with fresh aerated Deer Park bottled spring water (Table 3), dolomite chips [CaMg (CO_3)] and small limestone ($CaCO_3$) rocks that were added for shelter and to increase water hardness. The environmental chamber was programmed for constant darkness and at a temperature of 10 - 11^{o} C.

Table 3. Chemical composition and other parameters for Deer Park natural spring water (taken from deerpark water.com /#/home /2011). Units are mg/L or ppm unless otherwise noted.

Parameters	Units
Barium	None Detected – 0.018
Bicarbonate	None Detected – 150
Calcium	0.86 – 44.1
Chloride	2.1 – 12.8
Fluoride	None Detected – 9.19
Magnesium	None Detected – 9.1
Nitrate (as N)	None Detected – 4.6
Selenium	None Detected – 0.0022
Sulfate	2.2 – 17.8
Alkalinity	None Detected – 150
Conductivity (umhos/cm)	16.9 – 328
Hardness (Calcium)	2.1 – 172
Total Dissolved Solids	13 – 159
pH (units)	5.3 – 7.9

All microcosms measured 20cm W X 27.5cm L X 7cm H (Figure 15). Each microcosm was filled with up to five cm of Deer Park bottled spring water and an average of 105 – 120 animals in each FBC and FBR. Amphipods were then fed fallen maple leaves aged in water, watercress leaves and roots (FBC only) and occasionally brine shrimp flakes. Once per week, 2/3 of the water was replaced and new food was added.

Figure 15. Live *G. minus* animals. Density 105 – 120 animals from FBR. Taken by Tamela L. Brown.

To determine sex ratios for adult populations each amphipod was sexed as a matured male, a matured female, or either a matured intersexed male or intersexed female. The criterion used to determine phenotypic categories are illustrated in a series of photographs identifying each phenotype possessing the secondary sexual characteristic: if individuals had genital papillae, and no oostegites they were considered a male, and if oostegites were present they were considered females. If females' oostegites presented hairs they were marked as matured females (Figure 16).

Other secondary sexual characteristic used to assist the classification of sex was gnathopod size. If the 2nd gnathopod was larger, compared with the female, they were considered male and if the 2nd gnathopod was smaller, compared with the male, they were considered female (Figure 17). The section below is a series of images (Figures 18 – 21) illustrating the secondary sexual characteristics of normal males, normal females, intersexed females, and fully matured intersexed females with brood. The images were completed utilizing a scanning electron micrograph (SEM) resulting in high-resolution images (SEI). The process for obtaining SE images follows in the methods section of the chapter.

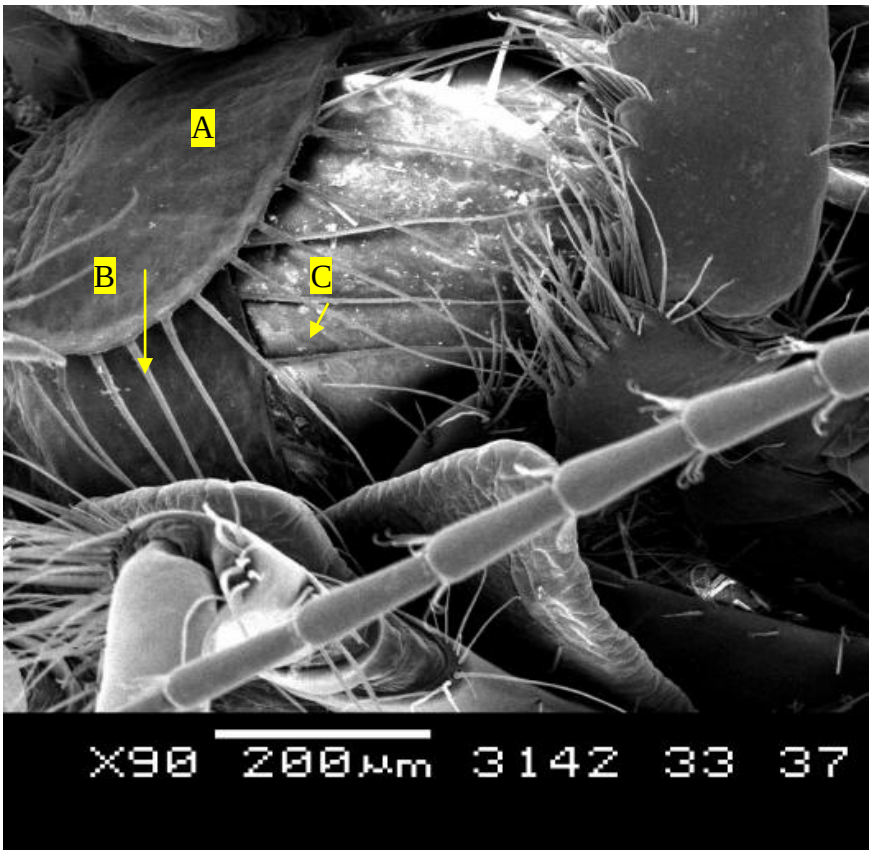

Figure 16. Scanning electron micrograph (Zeiss EVO MA10) of ventral side of normal female *G. minus* displaying mature brood plates with setae. Brood Plate (A), Brood Plate Setae (B) and Embryo (C).

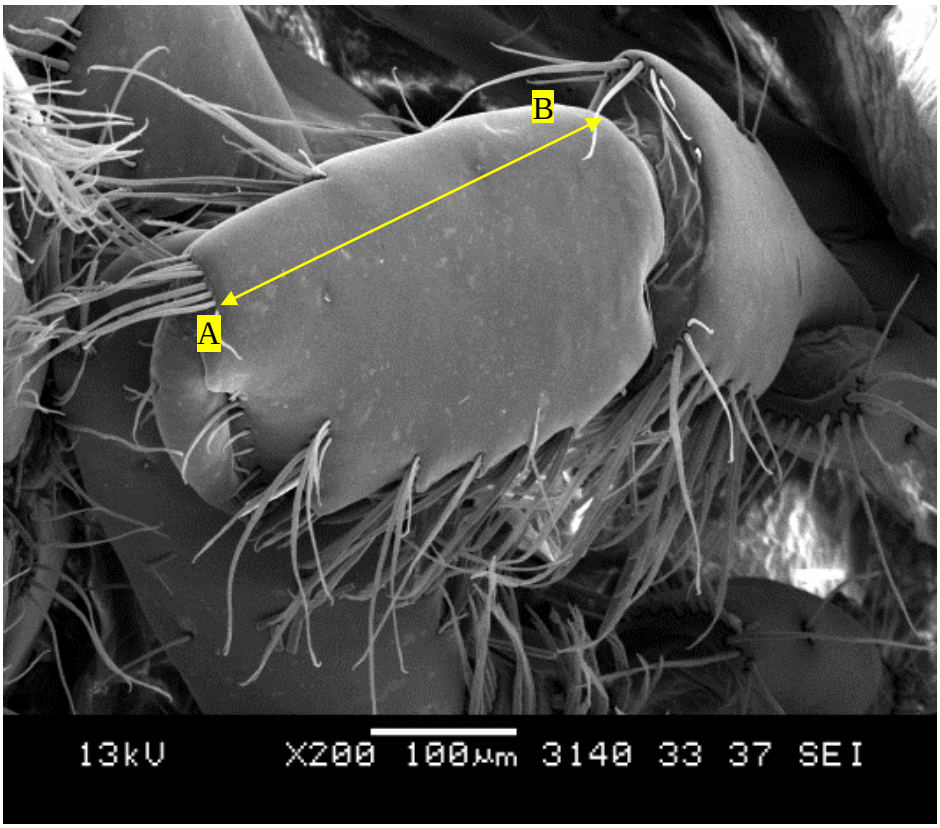

Figure 17. Scanning Electron Micrograph (Zeiss EVO MA10) of *G. minus* matured normal female 2nd gnathopod. Length of gnathopod without the claw from A to B is approximately 300 µm.

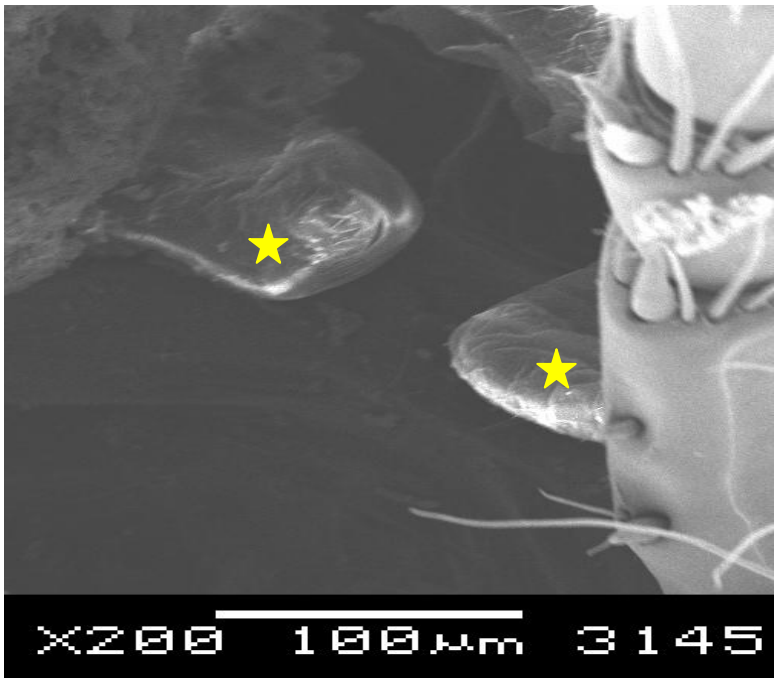

Figure 18. Scanning Electron Micrograph (Zeiss EVO MA10) of *G. minus* matured intersexed female displaying 2 papillea (star).

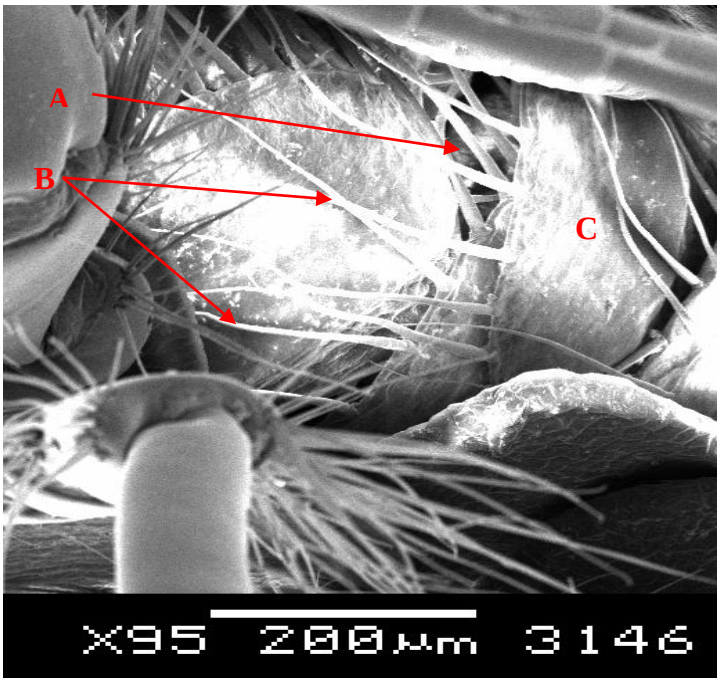

Figure 19. Scanning Electron Micrograph (Zeiss EVO MA10) of *G. minus*. Ventral side of matured intersexed female displaying embryo (beneath brood plate) (A), brood plate setae (B) and brood plate (C).

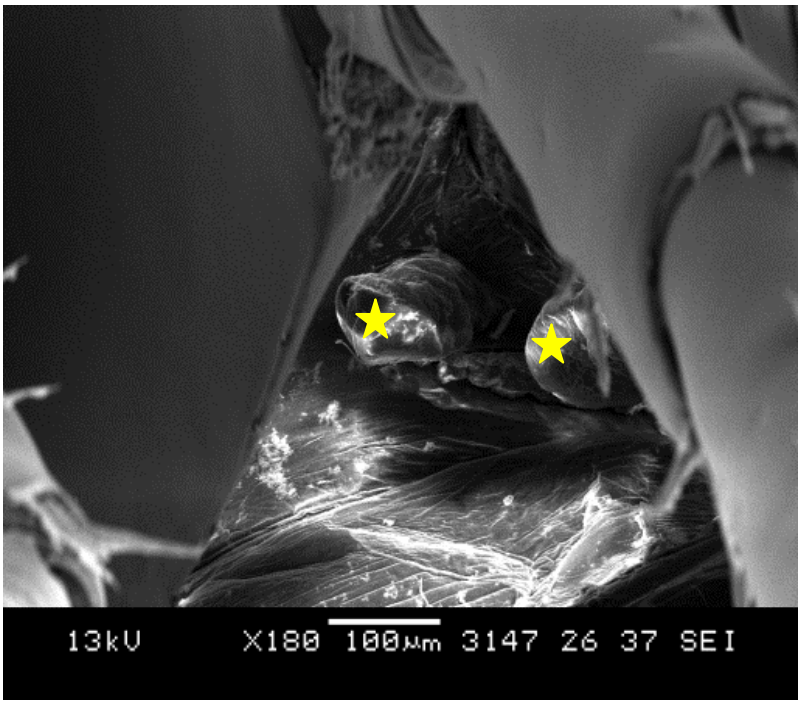

Figure 20. Scanning Electron Micrograph (Zeiss EVO MA10) of *G. minus*. Matured male displaying two papillae (yellow stars).

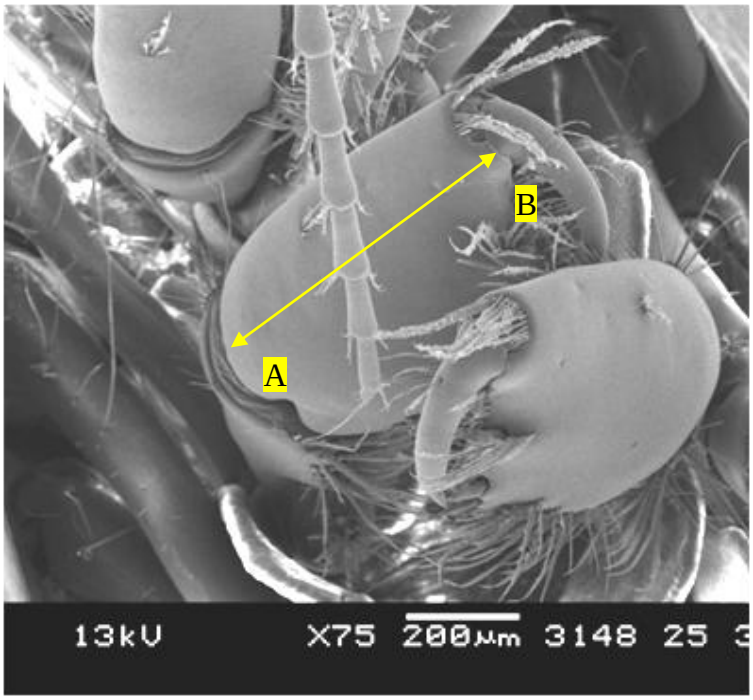

Figure 21. Scanning Electron Micrograph (Zeiss EVO MA10) of *G. minus* male 2nd claw w/hook. Length of claw (A to B) 600 µm.

To record body length, each individual amphipod was placed onto a petri dish with forceps and was gently pushed up next to a metric ruler (1 millimeter increments) until the dorsal side of the body was straightened. Care was specifically utilized for all preserved specimens as they were less flexible and could break. The body length was then measured, from the base of the first antenna to the base of the telson; length was recorded within ±1mm (Figure 22.).

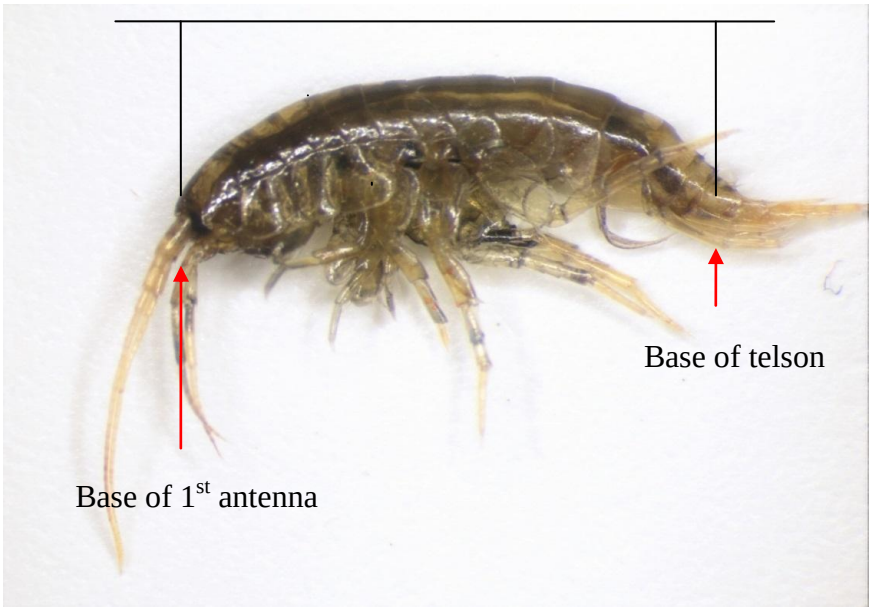

Figure 22. *G. minus* body length. Each amphipod measured from the base of the first antenna to the base of the telson. Specimen was pushed straight upwards (red arrows) against a metric ruler and the measurement for body length ±1mm was recorded. (Camera calibration 0.005mm/pixel; pic dimensions 2560 x 1920; Picture by Tamela L. Brown 12/12/15).

To compare and contrast populations, specimens were taken from the surrounding areas; Bradley Run, Montgomery County, Virginia USA (August 2013) and, Cedar Cave Spring, Adams County, Ohio USA (June 2014). Specimens from Antietam Creek Spring, Washington County, Maryland USA (2012), were kindly provided by Daniel Fong, PhD, Associate Professor, Department of Biology, American University, Washington, D.C. USA. Findings by Miller (1977) and Haley (1997) will also be used to compare with this study's findings regarding intersex prevalence.

3.3.3 Environmental measurements

During all sampling events, water quality parameters (pH, dissolved oxygen, temperature) were analyzed with a Vernier Lab Quest (Logger Pro 3) and sensors (Optical Dissolved Oxygen; Tris-Compatible Flat pH; Easy Temp for water and air) and as a backup instrument a hand-held YSI R650 MDS (SN061009987) was utilized with the appropriate probes (EXO Temperature Smart; pH Smart; Optical Dissolved Oxygen Smart); hardness and alkalinity were submitted to The Ohio State University (Soil and Water Quality Lab) for analysis according to Standard Water Quality Methods.

Prior to each sampling event and on site, instruments were calibrated appropriately and as follows: pH probe (2 pt method with pH 7 and 4 buffers), DO probe (water-saturated air calibration method).

Daylight hours were obtained to determine photoperiod (https://www.esrl.noaa. gov/gmd/ grad/solcalc/ sunrise.html. Retrieved 8/2018).

3.3.4 Data analysis

Relationships between biological parameters (*G. minus* body length and sex ratio) and environmental data were examined using Pearson's Correlation and multiple regression analysis (Bonferroni correction and confidence intervals). ANOVA and Tukey post hoc test were utilized to determine if the physical characteristics (dissolved oxygen, pH, water and air temperature, alkalinity and hardness) of populations sampled were significant (sex ratios and body lengths). Trend analyses regarding the three studies of FBC were conducted to determine changes and/or patterns of water characteristics.

All analyses and graphs were conducted using either Excel or Minitab 17.1 statistical software. After all data were determined to either be parametric or nonparametric the appropriate analysis, such as chi-squared Goodness of Fit, Mann-Whitney and Pearson's product - moment correlation coefficients tests were used as appropriate followed by Bonferroni Correction.

3.4 Results

3.4.1 Falling Branch Road *Gammarus minus*

Of 1,348 specimens collected from FBR 829 were males, 519 were intersexed females, and zero were normal females; the % of female intersexes varied from 31 – 47% of the total population and 100% of total female population (Table 4). The female populations peaked during the winter months (Nov – Feb). The % of males varied from 55 – 71% of the total population and matured males peaked during the fall months of 2011 and 2012.

Table 4. Falling Branch Road Amphipod Collections from 2010 – 2013. *G. minus* amphipods collected from FBR from Sep 2010 – Sep 2013. A total of 1,348 specimens were taken over the 17 month time frame; males = 829 and intersexed females = 519. There were no normal females or intersex males.

Site	**Year**	**Month**	**n**	**Normal Males *(n)***	**Intersex Males *(n)***	**Normal Females *(n)***	**Intersex Females *(n)***	**Total % Intersex**	**Total % Females Intersex**
FBR	2010	Sep	81	45	0	0	36	42.8	100
FBR	2010	Nov	84	46	0	0	38	45.2	100
FBR	2011	Feb	111	64	0	0	47	42.3	100
FBR	2011	May	108	70	0	0	38	35.2	100
FBR	2011	Jul	86	47	0	0	39	45.3	100
FBR	2011	Sep	82	51	0	0	31	37.8	100
FBR	2011	Nov	85	54	0	0	31	36.5	100
FBR	2012	Feb	102	65	0	0	37	36.3	100
FBR	2012	Jun	86	47	0	0	39	45.3	100
FBR	2012	Oct	80	47	0	0	33	41.2	100
FBR	2012	Nov	103	64	0	0	39	37.8	100
FBR	2013	Mar	100	58	0	0	42	42	100
FBR	2013	May	103	66	0	0	37	36	100
FBR	2013	Jul	122	87	0	0	35	28.7	100
FBR	2013	Sep	96	63	0	0	33	34.4	100

3.4.2 Body Lengths

Mean body lengths of *G. minus* from September 2010 to September 2013, for each phenotype, were recorded in Table 5. Male body lengths ranged from 6.1 to 8.7 mm and intersexed female body lengths ranged from 5.9 to 6.9 mm. FBR males were consistently larger than intersexed females regardless of the month. Mean body lengths for males and intersexed females were significantly different (ANOVA: F = 40.46; d.f. = 1; p =3.84E-07 *@ p-value 0.05*) between phenotype, but males and females were not significant from month to month (ANOVA: F = 1.56; d.f. = 10; p = 0.304 *@ p-value 0.05*).

Table 5. *G. minus* mean male and intersexed female body lengths collected from Falling Branch Road as measured and recorded from Sep 2010 to Sep 2013 to the nearest ±1mm. Confidence intervals for FBR populations' body lengths (Male 7.351, 7.955; Intersex Female 6.0153, 6.6200).

Year	Month	FBR Males	FBR Intersexed Females
2010	Sep	8.5	6.3
	Nov	8.7	6.9
2011	Feb	7.4	6.4
	Apr	7.8	6.7
	Jun	7	6.1
	Sep	8.5	6.6
	Nov	7.3	6.3
2012	Feb	8.4	6.3
	Apr	8.4	6.1
	Jun	6.7	6.1
	Aug	6.9	5.9
	Oct	7.4	6
	Dec	8.4	6.6
2013	Mar	8.5	6
	May	6.8	6
	Jul	6.1	6.9
	Sep	7.3	6.2

The proportion of males and intersex females' sub populations in FBR were both significant with photoperiod (Pearsons' coefficient = +0.637; p = 0.011 and -0.563; p = 0.029, respectively *@ p-value 0.04*). The percentage of males increased as photoperiod increased and the percentage of intersex females decreased as photoperiod increased (Figure 23). Spring temperature was not taken into account for this photoperiod analysis.

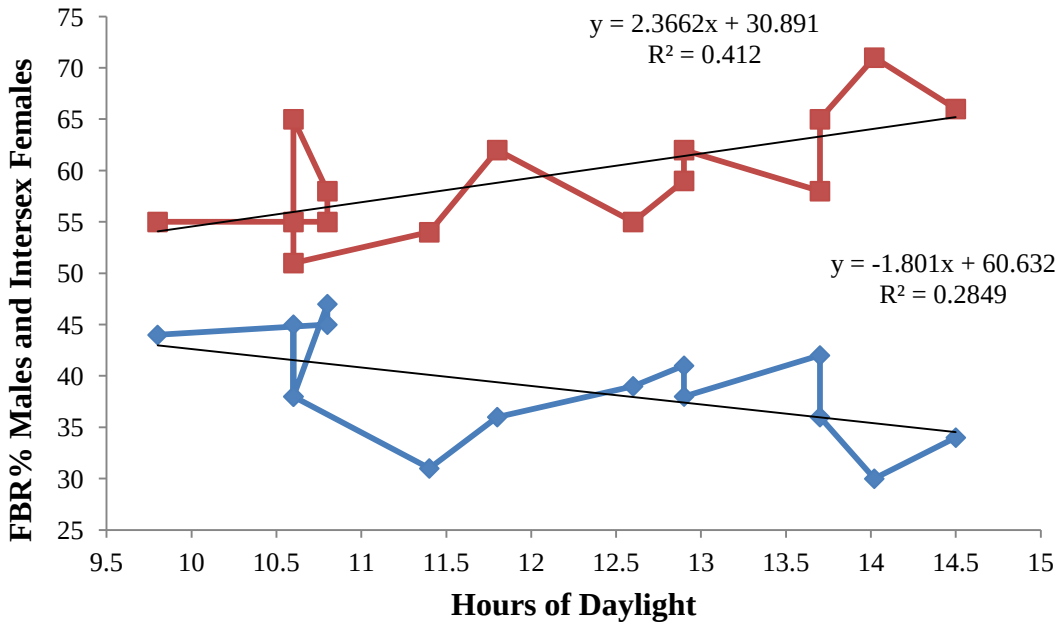

Figure 23. Falling Branch Road % Males and Intersexed Females Versus Hours of Daylight. The red line represents % males and the blue line represents % intersex females. There were no normal females observed. Males and intersex females were significant with photoperiod (Pearsons' coefficient = +0.637; P = 0.011 and -0.563; P = 0.029, respectively @ Bonferroni Corrected Critical p-value 0.04). Hours of daylight (https://www.esrl.noaa .gov/ gmd/ grad/solcalc/ sunrise.html retrieved 1/2018).

3.4.3 Falling Branch Cave *G. minus* Sex Ratios

In FBC, the proportions of males, females and intersexes were recorded bimonthly from Sept 2010 to Sept 2013 and summarized in Table 6. Of the 1,613 organisms from FBC, there were 929 males, 231 normal females, and 453 intersexed females. The overall population was consistently male biased during all months of each year; male ratios peaked (~68%) in March 2013, and dropped (~52%) during the summer months (June and July) of each year; intersexed and normal females ratios varied from 20 – 34% and 9 -19%, respectively.

Table 6. Falling Branch Cave (FBC) spring *G. minus* amphipods collected from Sep 2010 to Sep 2013. Classified by site, year, month, number, phenotype and per centages of intersexed organisms.

Site	**Year**	**Month**	***(n)***	**Males**	**Females**	**Normal Females**	**Intersex Females**	**Total % Intersex**	**Total % Females Intersex**
FBC	2010	Sep	79	45	34	15	19	24	56
FBC	2010	Nov	79	46	33	15	18	23	55
FBC	2011	Feb	53	30	23	5	18	34	68
FBC	2011	May	100	61	39	10	29	29	75
FBC	2011	Jul	96	50	46	15	31	33	72
FBC	2011	Sep	77	43	34	11	23	30	88
FBC	2011	Nov	77	43	24	12	22	29	83
FBC	2012	Feb	141	83	58	19	39	27	47
FBC	2012	Apr	99	61	38	10	28	28	74
FBC	2012	Jun	93	50	43	13	30	32	75
FBC	2012	Oct	138	80	58	20	38	28	48
FBC	2012	Nov	127	71	56	16	40	31	55
FBC	2013	Mar	107	73	34	13	21	20	59
FBC	2013	May	121	63	58	21	37	31	53
FBC	2013	Jul	115	67	48	14	34	30	63
FBC	2013	Sep	111	63	48	15	33	30	63
FBC	2013	Dec	102	67	35	13	22	21	60

The highest percentage of intersex females (78%), with reference to total females collected was in Feb. 2011 and the lowest in Sep. 2010 (55%). During the initial sampling months (Sep, Nov of 2010 and Feb of 2011) there were possible identification errors between smaller males and intersex females, which may contribute to the fluctuating sex ratios. For the total months of sampling, females displaying intersexuality were fairly static (65 – 75%), which perhaps indicates a more accurate sex ratio.

In FBC, within each year (2010, 2011, 2012 and 2013), the number of intersex females collected was significantly greater than the number of normal females collected (ANOVA: $F_{1,3}$ 14.88, p = 0.031 and Tukey-Kramer *@ p-value 0.05*). When females for each season of each year were analyzed, proportions of normal and intersexes varied, but did not differ significantly.

For further analysis, females were pooled by phenotype (intersex females and normal females) and season as follows: winter (Dec, Jan, Feb), spring (Mar, Apr, May), summer (Jun, Jul, Aug) and autumn (Sep, Oct, Nov). The proportions of normal females, and intersex females were compared across the years 2010 – 2013, and for each season of each year, proportions of normal and intersexes varied, but did not significantly differ, indicating sex ratios had remained stable (Chi-square X^2 = 5.89, df = 3, p = 0.65*;* Chi-square X^2= 5.501, d.f. 15, p = 0.98, 18 *@ p-value 0.05,* respectively). Figure 24 A, B, C, D.

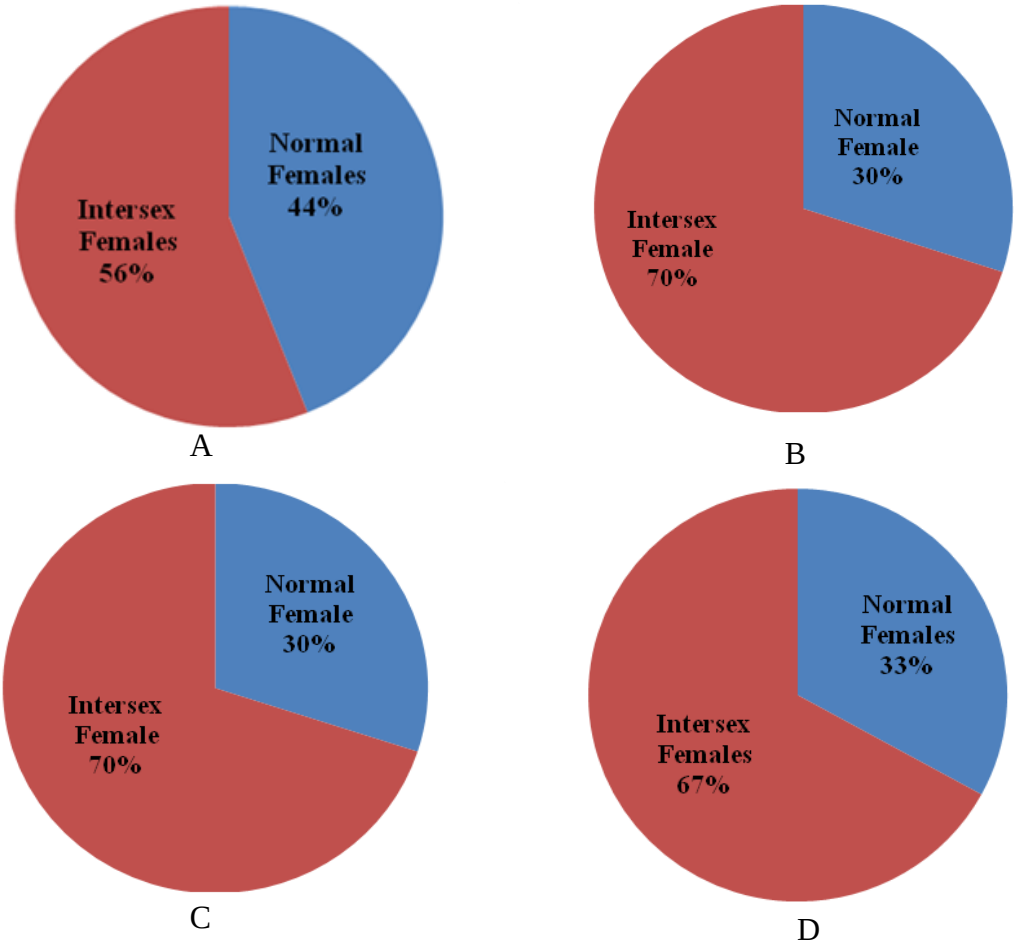

Figure 24. Yearly proportions of *G. minus* females (normal and intersex) collected bimonthly (2010 – 2013) from Falling Branch Cave. (A) 2010 Female sex ratios, total n = 67; normal females (*n* = 30); intersex females (n = 37). Intersexed females (56%) and normal females (44%); (B) 2011 Female sex ratios, total *n* = 176; normal females (*n* = 53); intersexed females (*n* = 123). Intersexed females 70% and normal females 30%. (C) 2012 Female sex ratios, total *n* = 253; normal females (*n* = 78); intersexed females (*n* = 175). Intersexed females 70% and normal females 30%. (D) 2013 Female sex ratios, total *n* = 188; normal females (*n* = 63); intersexed females (*n* = 125). Intersexed females 67% and normal females 33%.

The proportion of intersex females' sub population in FBC was significantly linear with photoperiod (Pearsons' Coefficient = +0.618; p = 0.011; *p-value 0.05*); as daylight hours increased, percentages of intersexed females and males increased. The % of FBC normal females were negatively correlated with photoperiod (Pearsons' Coefficient = -0.429; p = 0.097 and -0.292; p = 0.272, respectively); as the hours of daylight increased, percentages of normal females decreased (Figure 25).

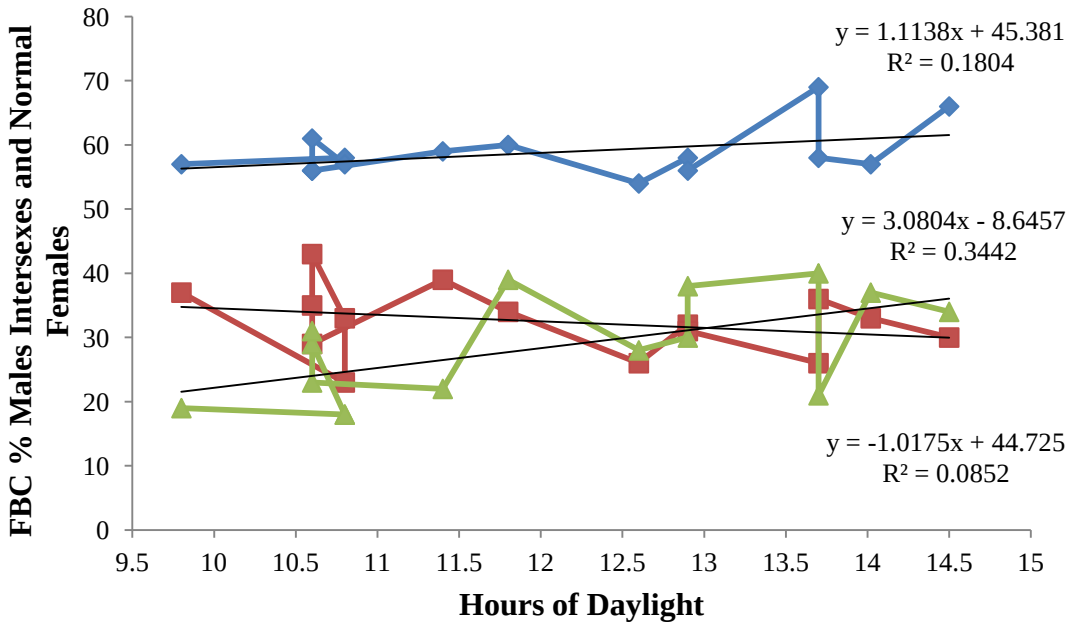

Figure 25. Falling Branch Cave % Males, % Normal Females and % Intersex Females versus Hours of Daylight. The blue line represents males, red line represents normal females and green line represents intersex females. Intersex females were significantly linear with photoperiod (Pearsons' Coefficient = +0.618; p = 0.011; *p-value 0.05*). Hours of daylight retrieved 1/2018 (https://www.esrl.noaa gov/gmd/ grad/ solcalc/ sunrise.html).

Body Lengths

Mean body lengths from September 2010 to September 2013, for each phenotype, were recorded in Table 7. Monthly mean body length of *G. minus* males ranged from 6.5 to 7.9 mm, normal females ranged from 5.1 to 5.3 mm, and intersexed females ranged from 5.5 to 6.5 mm. Male and intersexed female body lengths peaked late summer into the winter months while normal female body lengths remained relatively consistent from month to month and year to year. An ANOVA General Linear Model, conducted to determine if there was a significant difference in mean body lengths within each phenotypic population from month to month resulted in no significant differences within male, normal female, and intersexed females sub-populations ($F = 0.22$, d.f. 10, $p = 0.97$; $F = 7.06$, d.f. 10, $p = 0.67$*;* and $F = 0.93$, d.f. 10, $p = 0.59$ *@ p-value 0.05*, respectively).

Table 7. Mean body length of Falling Branch Cave Spring. *G. minus* males, normal females and intersexed females of each sampled month and year. All were measured from the base of the first antenna to the base of the telson in millimeters (± 1mm). Mean confidence intervals for FBC body lengths (Male 6.955, 7.245; Normal Female 5.0611, 5.3507; Intersex Female 5.8023, 6.0919), at pooled SD 0.30.

Year	Month	FBC Males	FBC Normal Females	FBC Intersexed Females
2010	Sep	7.1	5.3	6.5
	Nov	7	5.2	6
2011	Feb	7	5.3	6
	Apr	6.7	5.2	5.6
	Jun	7.9	5.2	6.1
	Sep	7.5	5.2	6
	Nov	7.6	5.2	6
2012	Feb	8	5.3	6
	Apr	6.8	5.2	5.8
	Jun	6.7	5.2	5.8
	Aug	7.1	5.1	6.2
	Oct	7.2	5.2	6.2
	Dec	7.3	5.1	6.2
2013	Mar	6.5	5.2	5.7
	May	6.7	5.3	5.5
	Jul	6.6	5.1	5.7
	Sep	7	5.2	5.8

An ANOVA revealed that males were significantly larger than intersexed females and intersexed females were significantly larger than normal females (F 17.48; d.f. 2; p = 0.01 *@ p-value 0.05*).

3.4.4 Prevalence of *G. minus* intersexes in similar springs

Comparable springs to FBC and FBR were Bradley Run, Cedar Fork Cave, and Antietam Creek. Three hundred and fifty six *G. minus* were collected and examined for the intersex condition to conduct a comparison of intersex prevalence. Of the total number of adults collected from Bradley Run (*n* = 120 adults), Cedar Fork Cave (*n* = 130 adults), and Antietam Creek (*n* = 106 adults) zero were found to be intersexed (Table 8).

Table 8. Intersex prevalence of *G. minus* found in sites comparable to FBC and FBR. The springs Bradley Run, Cedar Fork Cave and Antietam Creek and the numbers of adults collected at each site. Sexual phenotype determined (based on Chapter 1 physical description of intersex and normal).

Sample Site	**Adults *(n)***	**Normal *(n)***	**Intersexes *(n)***
Bradley Run, Montgomery County, Virginia USA	120	120	0
Cedar Fork Cave, Adams County, Ohio USA	130	130	0
Antietam Creek, Washington County, Maryland USA	106	106	0
Totals	356	356	0

3.4.5 Trends between FBC and FBR

The magnitude of the variability between mean body lengths for males and intersexed females, within each stream, appeared to be larger within FBRs' population (Figure 26).

Statistical analysis included ANOVA's, ANCOVA's with Tukey-Comparison, individual T-test, Pearson's Correlation-Coefficient, and Bonferroni Correction where applicapable.

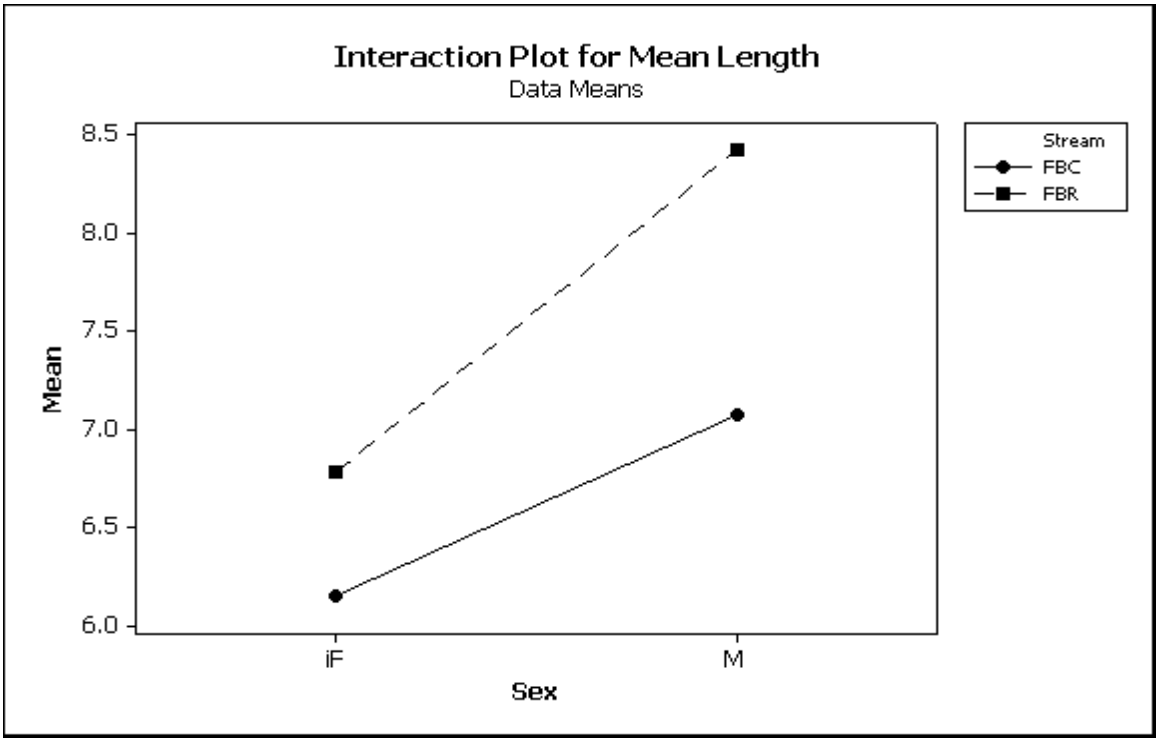

Figure 26. The magnitude of mean body lengths. FBR males and intersexed females appear larger than the magnitude of variability between males and intersexed females within the FBC population. (Minitab 17 Interactions Plot).

The following combinations resulted in significant differences for all mean body lengths (FBC normal females vs. FBC intersexed females; FBC normal females vs. FBR intersexed females; and FBC intersexed females vs. FBR intersexed females; T-test p = 0.001 *@ p-value 0.*05; T-test p = 0.001 *@ p-value 0.05;* and T-test p-=0.001 *@ p-value 0.05*), respectively (Figure 27).

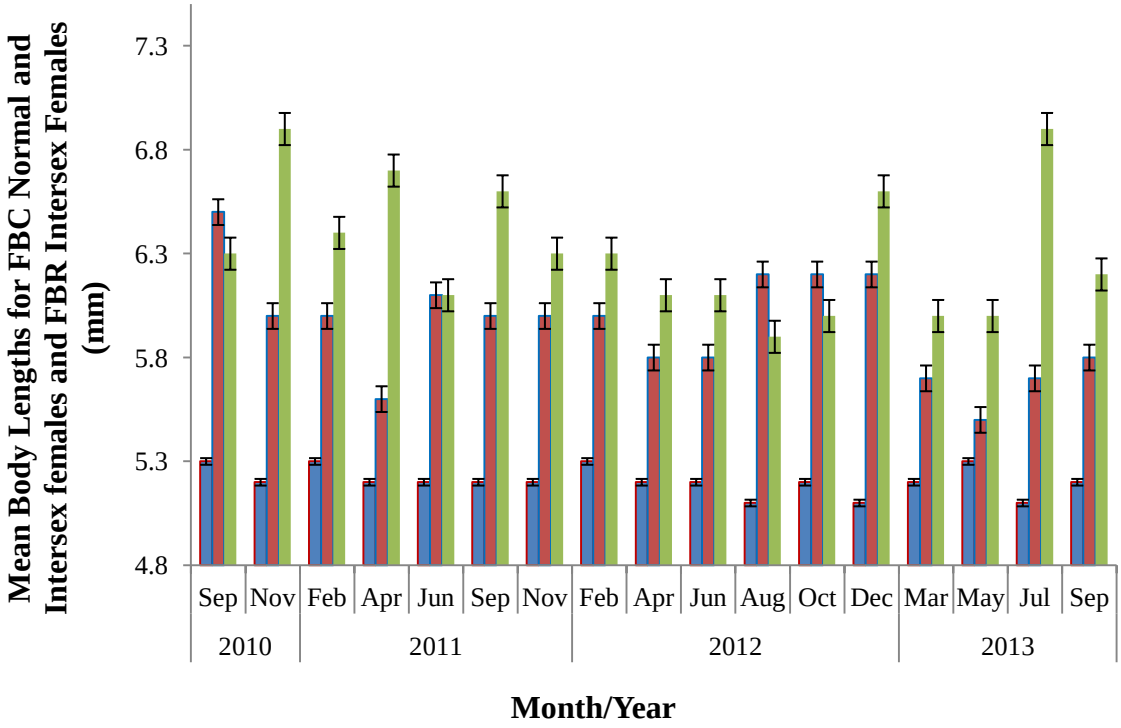

Figure 27. Mean Female Body Lengths for months collected. Reveals the variability of body lengths for all females in FBC and FBR. FBC and FBR intersexed females (red and green, respectively) illustrated the extreme variability of mean body lengths for each population over the sampled time frame. FBC normal females (blue) appeared much less variable and more stabilized within their population over the same time frame as FBC and FBR intersexed females.

The mean body lengths (mm) for all females were analyzed utilizing a bar graph (Figure 27) which illustrated the variation and trends within and between each population. FBC normal females showed less variability from month to month and year to year in body length; FBC intersexed females had more extreme highs (6.5 mm, Sep 2011) and lows (5.5 mm, May 2013) and appeared to follow a trend of larger body sizes in the late fall to winter and smaller body sizes in spring to summer. FBR intersexed females showed even more variability from a high of 6.9 mm to a low of 5.6 mm in Apr 2011.

Means of all female body lengths (mm) for both FBR and FBC were pooled to represent the four seasons as follows; winter (Dec, Jan, Feb), Spring (Mar, Apr, May), Summer (June, July, Aug) and Autumn (Sep, Oct, Nov) (Figure 28).

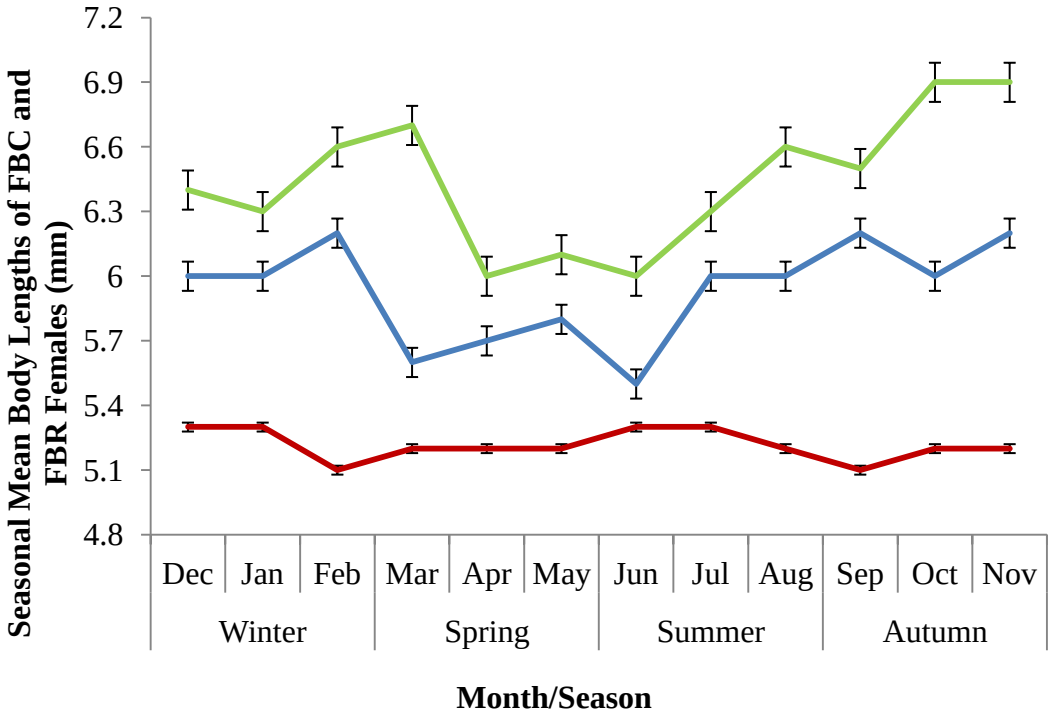

Figure 28. Mean body lengths of females (mm) from Falling Branch Cave and Road Springs by season. FBC normal females (red line), FBC intersex females (blue line), and FBR intersex females (green line). FBC and FBR intersex females growth patterns appear similar.

FBR intersexed females appeared to follow a similar pattern as FBC intersexed females. Larger body sizes from fall to winter, and smaller body sizes from spring to summer. FBC normal females remain static even when pooled by season.

3.6. Environmental Monitoring Data

3.6.1 FBC

Within FBC, the strongest correlation between sex ratio and environmental parameter was negative but not significant for intersex females and pH (Pearsons coefficient = -0.41, p = 0.12 @ *p-value 0.01*); the correlation indicates a negative direction that when pH increases intersex female sex ratio decreases, and vice versa. The weakest correlation was between normal females and pH and was not significant (Pearsons coefficient = -0.03, p = 0.92 @ *p-value 0.01*); the coefficient indicates that when pH increases normal female sex ratio decreases and vice versa. Environmental conditions are summarized in Table 9.

Table 9. Mean Measurements of Environmental Parameters. All measurements taken at time of collection of *G. minus* from Falling Branch Road and Falling Branch Cave sample sites.

Year /Mo	**Air Temp °C**	**Stream Temps °C**		**DO (mg/L)**		**pH**		**Alkalinity ($CaCO_3$ mg/L)**		**Hardness ($CaCO_3$ mg/L)**	
		FBC	FBR	FBC	FBR	FBC	FBR	FBC	FBR	FBC	FBR
2010 /S	12	13	12.5	8.5	9.2	7.8	7.6	320	211	361	250
2010 /N	10	13	12.5	8.2	. 2	7.6	7.6	319	212	360	250
2011 /J	12	12	9.2	9	10	7.6	7.5	322	212	360	245
2011 /M	20	14	4.6	8.5	9.6	7.1	7.7	298	263	362	270
2011 /M	22	13	9	8	9.4	7.2	7.8	320	225	361	200
2011 /J	29	14.5	10	8.7	9	7.4	7.4	327	225	322	201
2011 /S	24	13	13	8	8.5	7.4	7.5	320	210	325	236
2011 /N	7	13	13	9.4	9.2	7.8	7.9	318	212	360	248
2012 /F	16	12	12	9.1	9.5	7.5	7.5	287	238	366	259
2012 /A	23	13	13	9	10	7.6	7.6	305	219	362	246
2012 /J	33	14	14	8	9.6	7.2	8	321	228	323	216
2012 /A	36	14	14	8.5	8.8	7	7.6	325	224	326	220
2010 /O	21	13	13	9	8	7.8	8	318	224	375	214
2012 /D	8	12	12	9.3	9	7.5	7.9	324	220	366	257
2013 /M	22	14	14	8.8	10	7.6	7.5	320	220	366	270
2013 /M	25	13	13	8.2	9.5	7.5	7.6	322	220	360	211
2013 /J	31	14	14	8	10	7.5	7.5	322	225	345	216
2013 /S	25	14	14	9.5	9.5	7.4	7.8	325	205	355	235

Based upon the pH, alkalinity and hardness of both streams, the sample sites are both defined as alkaline hard-waters. Even so, on average and over the entire study period (2010 – 2013), the Falling Branch Cave sample site, was warmer with significantly lower dissolved oxygen ($p = 0.005$) increased alkalinity ($p = 0.005$) and greater hardness than FBR ($p = 0.005$) all at *p-value 0.01* (Table 10).

Table 10. Environmental Parameters for FBC and FBR. The results of t-test for all of the environmental parameters measured between FBC and FBR. Three of five environmental parameters measured (DO, alkalinity and hardness) were all significantly different between the two springs DO was significantly less in FBC than FBR (p = 0.005); alkalinity was significantly less in FBR than FBC (p = 0.005); and hardness was significantly less in FBR than FBC (p = 0.005). Stream temperatures (0.225) nor pH (0.045) were significantly different.

Sample Site	**Statistical Analysis**	**Environmental Parameter**	**Mean**	**Standard Deviation**	**DF**	**Bonferroni Corrected P-values**
FBC	t-test	Water Temp	13.2	±0.77	22	0.225
FBR		(^{o}C)	12.21	±1.95		
FBC	t-test	DO	8.65	±0.12	33	*0.005
FBR		(mg/L)	9.33	±0.54		
FBC	t-test	pH	7.5	±0.055	32	0.045
FBR			7.7	±0.044		
FBC	t-test	Alkalinity	317.4	±2.4	32	*0.005
FBR		($CaCO_3$ mg/L)	221.8	±3.1		
FBC	t-test	Hardness	353.1	±4	31	*0.005
FBR		($CaCO_3$ mg/L)	235.8	±5.3		

(*Bonferroni significance @ *p-value 0.01*)

A separate T-test for each parameter between FBC and FBR was conducted to compare the streams. Three of five parameters were significantly different as follows: DO was significantly less in FBC than FBR ($p = 0.005$); alkalinity was significantly less in FBC than FBR ($p = 0.045$); hardness was significantly less in FBR than FBC ($p = 0.005$). Neither stream temperatures nor pH were significantly different ($p = 0.225$ and 0.045, respectively).

Over a span of 43 years, Falling Branch Cave has undergone several noteworthy changes, which appear to affect the following environmental parameters; pH, DO, alkalinity and hardness. Falling Branch Cave is the only stream that had been studied by the three different researchers (Brown, Miller and Haley) therefore; environmental parameters recorded during each of the studies for the following months; May, June, Aug., Oct., Dec. and March. Pooling the same months from each study would increase the likelihood of proper representation, but since measurements were not conducted year to year it is not possible to know all of the changes that may have occurred in FBC (Table 11).

There were significant differences found between the environmental conditions between the three studies (ANOVA d.f. 6; F = 14.8; p = 0.0002 *@ p-value 0.05*). The following parameters were significantly different; pH conditions were significantly less acidic between Haley and Brown (ANOVA d.f. 2; F = 14.79; p = 0.001 *@ p – value 0.05*) and Tukey test, alkalinity for Haley was significantly lower than Miller (ANOVA, d.f. 2; F = 3.59; p = 0.049 *@ p–value 0.05*) and Tukey test; hardness ($CaCO_3$ mg/L) was also significantly lower for Haley than Miller (ANOVA, d.f. 2; F = 4.24; p = 0.031 *@ p-value 0.05*) (Table 12).

Table 11. Environmental measurements from Miller (1977), Haley (1997) and Brown (2013) for Falling Branch Cave Spring. Data pooled over same months (May, June, Aug., Oct., Dec. and March).

Years of Each Study	**Stream Temperature (^{o} C) Means and SD**	**Stream pH Means and SD**	**DO ($CaCO_3$ mg/L) Means and SD**	**Alkalinity ($CaCO_3$ mg/L) Means and SD**	**Hardness ($CaCO_3$ mg/L) Means and SD**
Brown 2010 – 2013	13.35 ±0.80	7.43 ±0.24	8.64 ±0.42	315.86 ±9.90	353.7 ±19.4
Haley 1995 - 1997	12.61 ±0.47	*7.91 ±0.16	9.31 ±0.56	*304.91 ±13.77	*327.9 ±15.4
Miller 1975 – 1977	13.57 ±1.04	7.84 ±0.12	8.82 ±0.90	320.6 ±5.92	359.4 ±24.3

(*Significance *p-value @ 0.05*)

The mean stream temperature measured in degrees Celsius was highest at 13.58 in 2013 and lowest at 12.61 in 1997. The difference in mean temperature measurements of Brown (2013) and Millers' (1977) when compared with Haley (1997) was most likely due to equipment fluctuations rather than an actual environmental change. There is a significantly strong decrease in pH over the time span of 43 years. The mean pH for 1977 was 7.84, for 1997 it was 7.97 and for 2013 it was 7.43. There was a significant correlation between pH and per cent of intersex females from 1977 (Miller) to 2013 (Brown) (Pearson's correlation coefficient = 0.48; p = 0.027 @ *p-value 0.05*). The overall trend was a linear relationship, as pH decreased FBC intersex females decreased. Dissolved oxygen was not statistically significant but there is a moderate decrease in DO over 43 years. The greatest value of DO was 9.31mg/L in 1985, was 8.83 mg/L in 1977 and 8.64 mg/L in 1977. The overall trend was a slightly linear relationship, as DO decreased FBC intersex females decreased.

The mean alkalinity measured in $CaCO_3$ mg/L was significantly different between Miller (320.57) and Haley (304.91). The highest measurement for mean alkalinity was 320.58 $CaCO_3$ mg/L and the lowest was 304.91 $CaCO_3$ mg/L. The overall trend was a linear relationship, as alkalinity decreased FBC intersex females decreased. The mean hardness measured in $CaCO_3$ mg/L was significantly different between Miller (359.43) and Haley (327.86). The highest measurement for mean hardness was 359.43 $CaCO_3$ mg/L and the lowest was 327.86 $CaCO_3$ mg/L. The overall trend was a linear relationship, as hardness decreased FBC intersex females decreased.

Table 12. Pearson's correlation coefficient analysis. FBR and FBC female mean body lengths (mm) correlated with environmental parameters. Falling Branch Road intersex females and FBC normal females were both negatively correlated with alkalinity, and FBC intersex females strongest correlation was with DO, but none were significant.

Statistical Test	**Env. Parameter**	**FBC Normal Females**	**FBC Intersex Females**	**FBR Intersex Females**
Pearsons Coefficient Correlation	Stream Temp.	0.116	0.023	-0.084
P---Value (Bonferroni corrected P - value)		0.657 (1)	0.931 (1)	0.749 (1)
Pearsons Coefficient Correlation, P-Value (Bonferroni corrected P - value)	pH	-0.428	0.187	-0.072
		0.087 (1)	0.471 (1)	0.785 (1)
Pearsons Coefficient Correlation, P-Value (Bonferroni corrected P - value)	Dissolved Oxygen	-0.270	-0.446	-0.238
		0.294 (1)	0.073 (0.876)	0.358 (1)
Pearsons Coefficient Correlation P-Value (Bonferroni corrected P - value)	Alkalinity	-0.495	0.062	-0.544
		0.043 (0.52)	0.812 (1)	0.024 (0.29)

(Bonferroni corrected critical *p-value @ 0.004*)

Falling Branch Cave normal females and FBR intersexed females were both negatively correlated with alkalinity (Table 12). Both were negatively correlated indicating that as alkalinity increased, body lengths decreased and vice versa. All the parameters may or may not have played a role in *G. minus* normal female body lengths, because three points of data cannot be a conclusive data set.

Table 13. Pearson's correlation coefficient analysis. Pooled data representing seasonality of FBR and FBC female phenotypes and mean body lengths (mm).

Season	Combinations	Pearsons' Correlation Coefficient, P-Value (Bonferroni Corrected P – Value)
Spring	FBC iF and FBR iF	-0.530, 0.470 (1)
Spring	FBC iF and FBC nF	0.309, 0.691 (1)
Spring	FBR iF and FBC nF	0.469, 0.531 (1)
Summer	FBC iF and FBR iF	0.500, 0.667 (1)
Summer	FBC iF and FBC nF	0.000, 1.000 (1)
Summer	FBR iF and FBC nF	-0.866, 0.333 (1)
Autumn	FBC iF and FBR iF	-0.839, 0.075 (1)
Autumn	FBC iF and FBC nF	0.312, 0.610 (0.9)
Autumn	FBR iF and FBC nF	-0.000, 1.000 (1)
Winter	FBC iF and FBR iF	0.981, 0.019 (1)
Winter	FBC iF and FBC nF	-0.426, 0.574 (0.23)
Winter	FBR iF and FBC nF	-0.418, 0.582 (1)

(Bonferroni corrected critical *p – value @ 0.004*)

Means of each phenotypes body lengths were analyzed using Pearson's Correlation Coefficient at *p-value 0.05* and Bonferroni Corrected Critical *p-value 0.004* (Table 13). The FBR intersex females mean body lengths (6.35 mm) during the winter season was positively correlated with the mean body lengths (6.0 mm) of FBC intersex females, but was not significant. This correlation indicates that the intersexed females from both streams (FBC and FBR) have similar molt and growth patterns, which notably contribute to the maturation cycles of each population.

3.6.2 FBC and FBR *G. minus* sex ratios and % of ovigerous females versus environmental parameters

Within FBC (Table 14), the strongest correlation between sex ratio and environmental parameters was negative, but not significant for intersex females and pH (Pearsons' coefficient = -0.41, Bonferroni corrected p = 1 *@ p-value 0.002*); the correlation indicates a negative direction that when pH increases intersex female sex ratio decreases, and vice versa. The weakest correlation was between normal females and pH and was not significant (Pearson's coefficient = -0.03, Bonferroni corrected p = 1 *@ p-value 0.002*); the coefficient indicates that when pH increases normal female sex ratio decreases and vice versa.

Table 14. Environmental parameters (pH, water temps in oC, alkalinity in CaCO3 mg/L, hardness in CaCO3 mg/L, and dissolved in oxygen mg/L). Correlated with sex ratios of *G. minus* and the means of environmental parameters from both sample sites (Falling Branch Cave and Road springs). All p's = 1; there was no significance @ p-value 0.002.

Statistical Test	**Env. Parameter**	**FBC Males**	**FBC intersex Females**	**FBC Normal Females**	**FBR Males**	**FBR Intersexed Females**
Pearsons coefficient	pH	-0.9	-0.41	-0.03	0.29	0.18
p-value		0.74	0.12	0.92	0.30	0.53
Bonferroni Corrected p		(1)	(1)	(1)	(1)	(1)
Pearsons coefficient	Water Temps	0.26	0.23	0.12	0.003	0.03
p-value		0.33	0.40	0.65	0.10	0.93
Bonferroni Corrected p		(1)	(1)	(1)	(1)	(1)
Pearsons coefficient	Alkalinity	-0.06	0.041	0.36	-0.34	-0.04
p-value		0.81	0.88	0.17	0.22	0.88
Bonferroni Corrected p		(1)	(1)	(1)	(1)	(1)
Pearsons coefficient	Hardness	0.04	-0.15	-0.10	-0.3	-0.15
p-value		0.90	0.56	0.71	0.27	0.58
Bonferroni Corrected p		(1)	(1)	(1)	(1)	(1)
Pearsons Coefficient	Dissolved Oxygen	0.19	0.12	-0.05	0.08	-0.38
p – value		0.48	0.65	0.85	0.79	0.16
Bonferroni Corrected p		(1)	(1)	(1)	(1)	(1)

(Bonferroni Corrected Critical *p-value 0.002*)

Within FBR (Table 14), the strongest correlation between sex ratio and environmental parameter was between intersex females and dissolved oxygen and was not significant (Pearson's coefficient = -0.38, Bonferroni corrected p = 1 @ *p-value 0.002*); the weakest correlation was between males and water temperatures and was not significant (Pearsons' coefficient = 0.003, Bonferroni corrected p = 1 @ *p-value 0.002*). All corrected for multiple comparisons (Bonferroni Correction).

Table 15. Pearson's coefficient and Bonferroni Correction *p-values* for both FBC and FBR environmental parameters (pH, water temps in ^{o}C, alkalinity in $CaCO_3$ mg/L, hardness in $CaCO_3$ mg/L) correlated to mean body lengths of *G. minus*. There was no signficance at *p-value 0.002*.

Statistical Test	**Env Parameter**	**FBC Males**	**FBC Intersexed Females**	**FBC Normal Females**	**FBR Males**	**FBR Intersexed Females**
Pearsons coefficient	pH	-0.19	0.08	-0.23	-0.19	-0.14
p-value		0.46	0.73	0.36	0.45	0.58
Bonferroni Corrected p		(1)	(1)	(1)	(1)	(1)
Pearsons coefficient	Water Temps	0.14	0.005	-0.15	-0.23	-0.42
p-value		0.57	0.98	0.57	0.38	0.09
Bonferroni Corrected p		(1)	(1)	(1)	(1)	(1)
Pearsons coefficient	Alkalinity	0.02	0.47	-0.31	-0	0.06
p-value		0.92	0.04	0.23	0.99	0.81
Bonferroni Corrected p		(1)	(0.8)	(1)	(1)	(1)
Pearsons Coefficient	Hardness	-0.28	0.03	-0.25	-0.37	-0.04
p-value		0.27	0.91	0.33	0.14	0.87
Bonferroni Corrected p		(1)	(1)	(1)	(1)	(1)

(Bonferroni Critical *p-value 0.002)*

Within FBC (Table 15), the strongest correlation was between mean body lengths of intersex females and alkalinity (Pearson's coefficient = 0.47; p = 0.04 @ *p-value 0.05)* (Figure 3.24). However, when Bonferroni Correction is applied, the significance falls away (*p-value 0.002*). The correlation of alkalinity and FBC intersex females indicates that the relationship would never be zero and as alkalinity increases, body length increases. The weakest correlation between mean body lengths and an environmental parameter was between intersex females and water temperature (Pearson's coefficient = 0.005, p = 0.98 @ *p-value 0.05*). The correlation coefficient implies there was no relationship between the water temperatures and intersex female amphipods mean body lengths.

Within FBR the strongest correlation was between intersex females and water temperatures (Pearson's coefficient = -0.42, p = 0.09 @ *p-value 0.05*). The correlation coefficient implies there was a negative correlation between intersex female body length and water temperature; as water temperature increased the female body length decreased and vice versa. The weakest correlation was between FBR male body length and alkalinity (Pearson's coefficient = -0.003, p = 0.99 @ *p-value 0.05*); there were no statistically significant relationships (Bonferroni corrected critical *p-value 0.002*). The correlation coefficient implies that there is no relationship between alkalinity and male amphipod mean body length.

Per cent ovigerous females and environmental data

Both, percentage of ovigerous females (FBC and FBR) and environmental parameters {stream temperatures (^{o}C); pH; DO (mg/L); alkalinity and hardness (mg/L $CaCO_3$)}, were pooled to analyze potential seasonal trends; Winter (Dec, Jan, Feb), Spring (Mar, Apr, May), Summer (Jun, Jul, Aug), and Autumn (Sep, Oct, Nov).

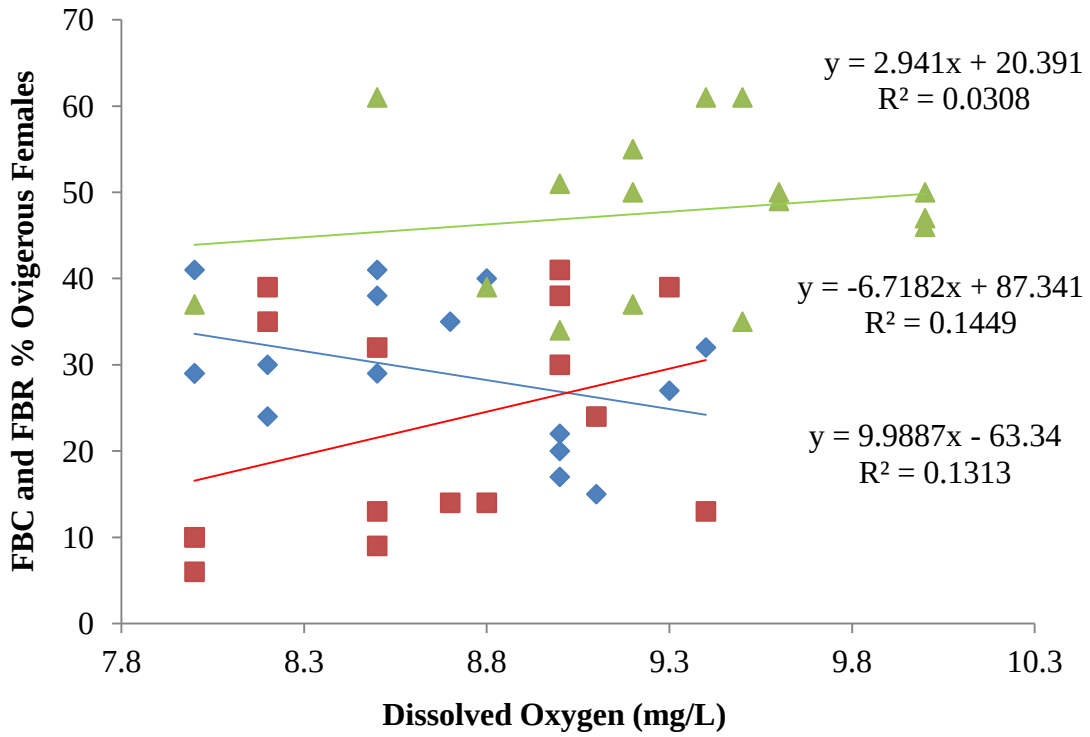

Figure 29. Falling Branch Cave and Road % Ovigerous Females Correlated With DO. Falling Branch Cave normal females are positively correlated with DO; FBC intersex females are weakly correlated with DO; FBR intersex females are negatively correlated with DO. FBR intersex females (green line), FBC intersex females (red line), FBC normal females (blue line). None were significant (Bonferroni Corrected *p-value 0.002*).

Seasonally, Falling Branch Road proportions of ovigerous intersexed females and DO were negatively correlated (Pearson correlation coefficient = -0.605; p = 0.013 @ Bonferroni Corrected *p-value = 0.002*); as DO increased, proportions of ovigerous intersexed females decreased. Falling Branch Cave proportions of ovigerous normal females was correlated with DO, as DO increased the proportion of ovigerous normal females increased. The FBC intersexed females resulted in zero correlation.

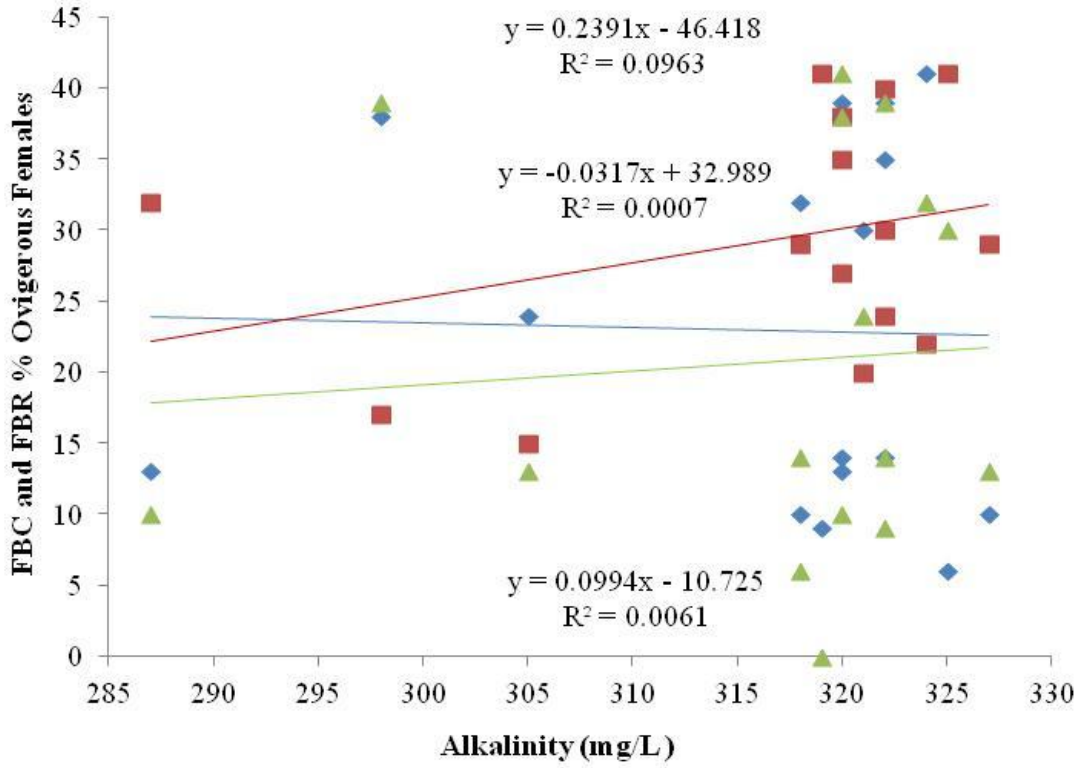

Figure 30. Falling Branch Cave and Road % ovigerous females correlated with respective stream alkalinity (mg/L $CaCO_3$) by seasons. Neither FBC normal (red line) or intersex females (blue line) are correlated with alkalinity. Intersex females in FBR (green line) are not correlated with alkalinity. There are no significant correlations (*p-value 0.002).*

Seasonally, FBC normal females, intersex females, and FBR intersex females were not considered correlated with alkalinity. There are no significant correlations (*p-value 0.002).*

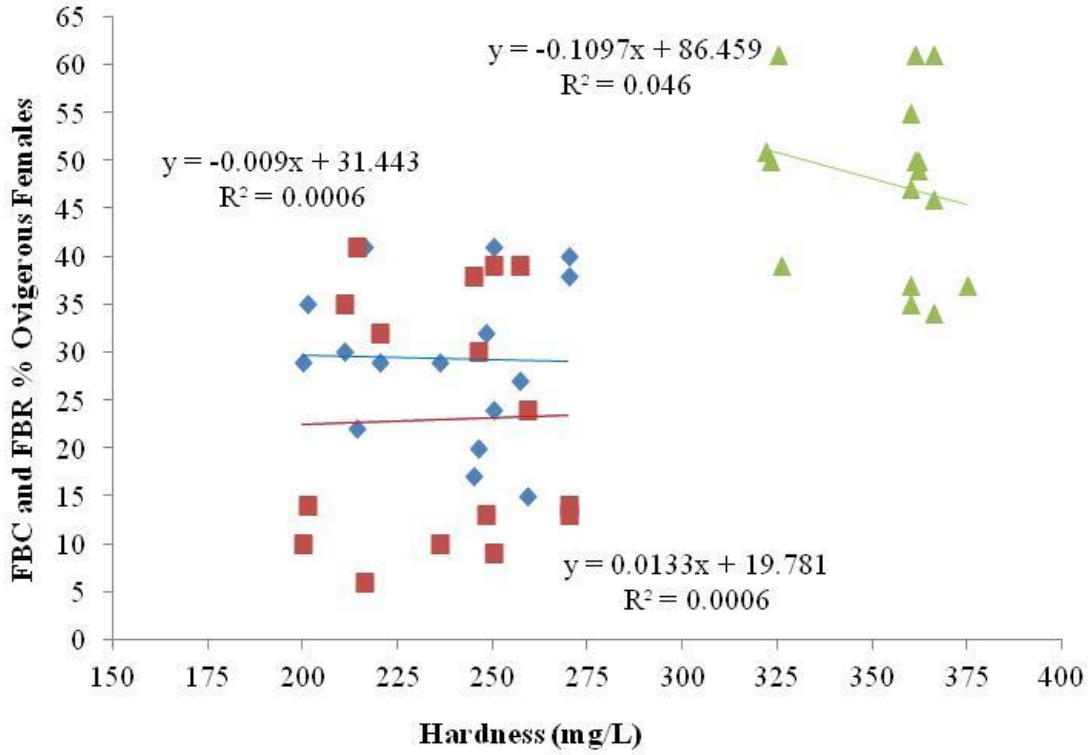

Figure 31. Falling Branch Cave and Road % ovigerous females correlated with respective stream hardness (mg/L $CaCO_3$) by season. FBC normal (red line) and intersexes (blue line) are not correlated, and FBR intersex females (green line) are negatively correlated, slightly.

Seasonally, hardness was not correlated with FBC normal females; FBR intersexed females were mildly correlated; as hardness increased, proportions of ovigerous intersexed females slightly decreased. There are no significant correlations (*p-value 0.002*).

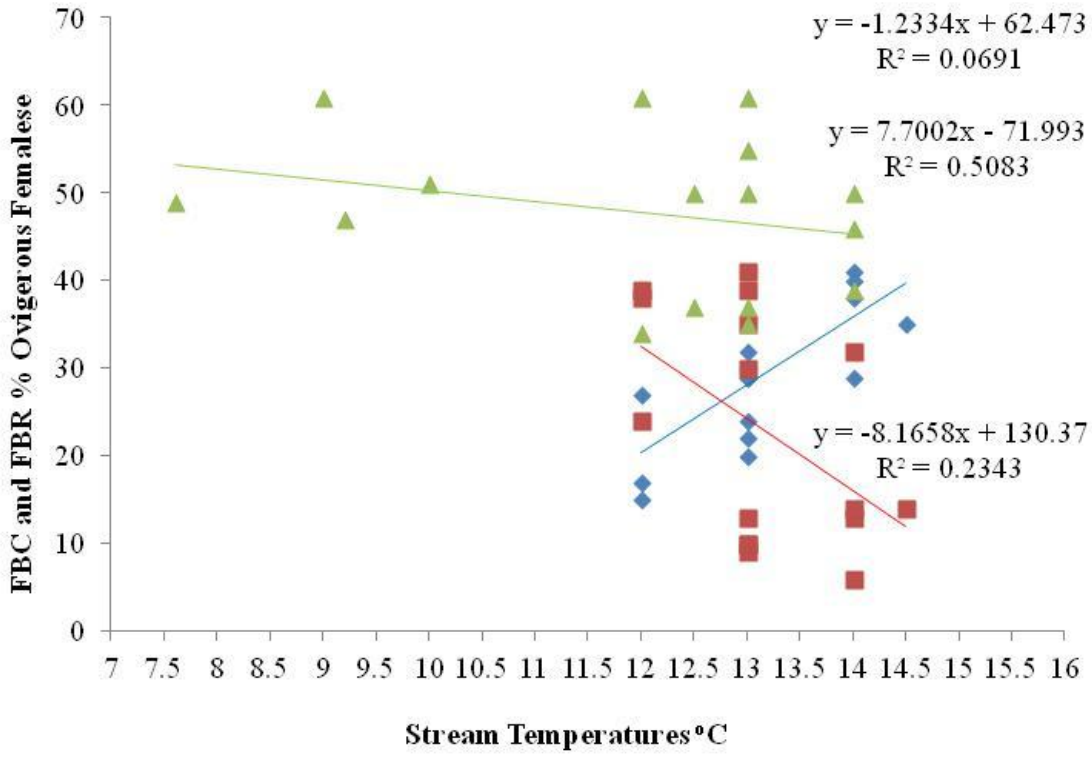

Figure 32. Falling Branch Cave and Road % ovigerous females correlated with respective stream temperature (^{o}C) by season. FBC intersex % ovigerous (blue line) females and stream temperatures were positively correlated; FBC normal females (red line) were negatively correlated but not significant. FBR intersex females (green line) are not correlated. There are no significant correlations (*p-value 0.002*).

FBC intersex % ovigerous (blue line) females and stream temperatures were positively correlated; FBC normal females (red line) are negatively correlated but not significant. FBR intersex females (green line) are not correlated. Thre are no significant correlations (*p-value 0.002*).

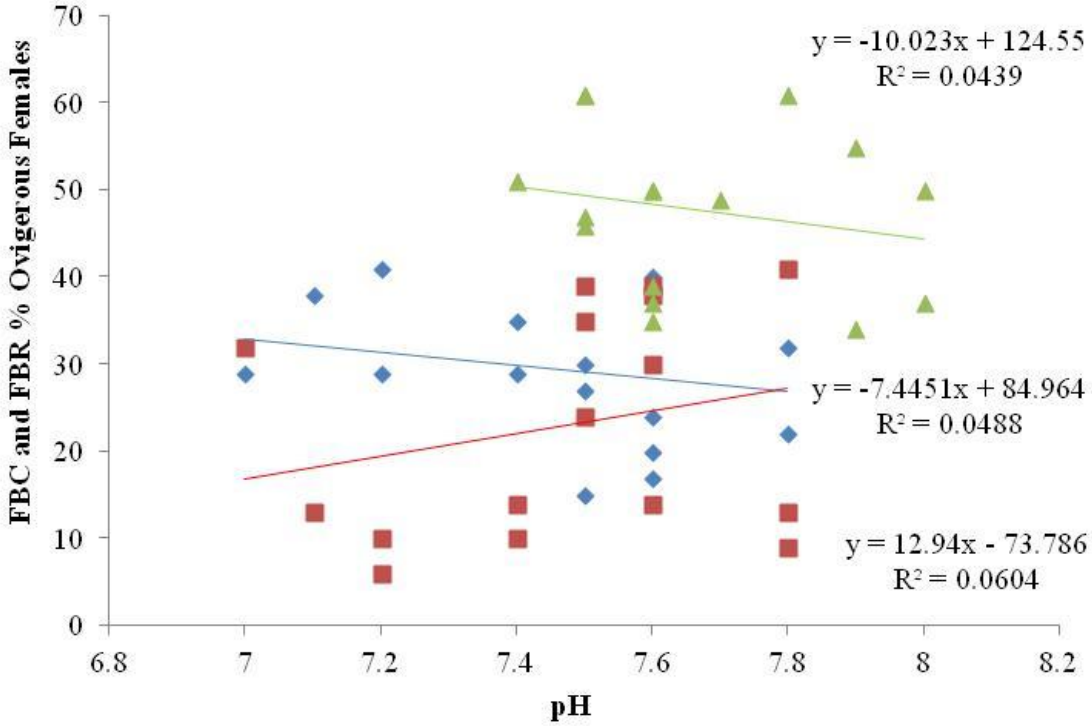

Figure 33. Falling Branch Cave and Road % ovigerous females correlated with respective pH by season. Both FBC and FBR % ovigerous intersex females and pH are not correlated (blue line and green line, respectively). FBC normal females (red line) and pH are not correlated. There are no significant correlations (*p-value 0.002*).

Seasonally, both FBC normal and intersex females; FBR intersex females were not correlated with pH. There are no significant correlations (*p-value 0.002*).

The data collected and results of the findings within this chapter will help to lay the ground work for the hypothesis of the remaining chapters.

3.7 Discussion

The intersex condition was first revealed in amphipods, from as early as the late 1800's (Sexton, 1906, 1921) and has continued to be found throughout the different species and populations of Amphipoda (Huxley, 1924; Sexton and Huxley, 1921; Bulnheim, 1975; Buikema et al., 1978; Miller, 1977; Buikema, 1980; Dunn et al., 1990; Ladewig et al., 2002; Glazier, 1999; Buikemia et al., 1980; Ford and Fernandes, 2005; Ford and Glazier., 2008; and Glazier et al., 2012). In this chapter, the intersexed condition found within the animal kingdom was defined; sex ratios of FBC and FBR were determined, maturation and the prevalence of intersexes in amphipod populations were analyzed, then subsequently compared and contrasted with other amphipod populations within the same geographical area. In addition, amphipod body lengths, sex ratios, proportions of ovigerous females and environmental parameters were all assessed for relationships.

The literature supports that the prevalence of intersexes in amphipod populations may vary by species, location and if known, causes (Dunn et al., 1990, 1993; Kelly et al., 2004; Hough et al., 1992; Ford et al., 2004; Ladewig et al., (2004). Intersexes have been reported from a number of Gammarids populations from both freshwaters and brackish waters that are documented by several studies where differing frequencies are found in separate locations. For example, *G. duebeni* intersexes are found in two different locations; one, in Bundle Bay, Northumberland where 10.8% of the sampled population is intersexed (Dunn et al., 1990), and secondly, *G. duebeni* populations found in Cumbrae, Scotland varied from 0.5 – 5.2% intersex (Kelly et al., 2004). At the time of Dunn's et al., (1990) observations, a population of 10.8% intersex was considered to be a high frequency (Dunn et al., 1990; Ladewig et al., 2004). In Germany (Lockwitzbach and Körsch), there are two streams that were investigated by Ladewig et al., (2004). Each stream was represented by two sample sites (one upstream and one downstream of a sewage treatment plant) where a total of 1,521 adult *G. fossarum* animals were taken; of the total 1,002 sampled from the Lockwitzbach site 8.8% were intersexed and of the 519 adults from the Körsch stream 0.03% were intersexed.

In most cases, intersexes have been reported in very small numbers, as in the mysid *Neomysis integer* (Hough et al., 1992). The samples were collected throughout the year 1988, from the Conwy Estuary in Northern Wales and seven out of 13,700 *N. integer* were intersexed. A total of 5,469 *E. marinus*, collected by Ford et al., (2004), from the east coast of Scotland, UK, resulted in the following subpopulations of intersexes; 0.024% (129:5469) intersexed males and 0.061% (333:5469) intersexed females. As the literature illustrates, the intersex condition in Amphipoda, while diverse, is relatively sparse within populations (Bulnheim, 1975; Glazier, 2008). The various causes of intersexuality that are recognized in the examples above include: microsporidia parasites and environmental sex determination in *G. duebeni* and *E. marinus* (Rogers-Gray et al., 2004; Dunn and Smith 1990; Dunn et al., 1993, Ford et al., 2004, respectively), pollution-induced in *G. fossarum* and *E. marinus* (Ladewig et al., 2004; Ford et al., 2004), and possibly genetic in the mysid *N. integer* (Ginsburger-Vogel & Charniaux-Cotton, 1982). While some causes of the intersexes condition are documented, the anomaly is still not completely understood and as previously mentioned, the prevalence of intersexuality is typically sparse. Nevertheless, the most notable exceptions include the amphipods from FBC and FBR, *G. minus* that are the subjects of the current investigations. The aim of the population field studies on Falling Branch Road was to investigate current conditions to determine if intersexes have varied over time (~3 years) and perhaps gain insight to the cause and persistence of these highly intersexed specimens.

Sex Ratios for FBC and FBR

In this study, both populations were male biased during all sampled months. Specifically, the sex ratio for the total sampled number of FBC (n = 1613) was as follows: males peaked in March 2013 (~68%) and dropped during the summer months of June and July (~52%), intersexed and normal females varied from 20 – 34% and 9 – 19%, respectively, as a proportion of the total mature animals. When the females were pooled by season, the intersexes and normal females were significant indicating that sex ratios between females had remained fairly static over this sampling period.

In FBC, the normal females subpopulation is considerably less variable by month as the extremes are only 10 units apart (9% in Feb. to 19% in Oct.); the subpopulation of intersexed females in FBC is more variable by month as the extremes are 14 units

apart (20% in March and 34% in Nov). The intersexes in FBC have diminished from 100% (Miller, 1977) to 60% (Buikema, 1980; Ford and Glazier, 2008; and Glazier et al., 2012). Normally, if intersexes are present because of an environmental parameter (e.g. temperature, photoperiod), parasitic influence (e.g. microsporidia) and/or pollution (e.g. endocrine disruption via pesticides) it may be reasonable to say that the decrease in numbers of intersexes is due to the absence of this influence.

Pattern differences in FBR may be due to the influx of intersex females into the population, which would present more available reproductive females to males causing peaks of intersexes. During early winter to summer, the proportion of intersex females increased (28 – 34%) and the proportion of males declined (68 – 52%); from late summer to winter, intersex females proportions decreased (32 – 25%) and proportions of males increased (52 – 68%). As previously discussed, the possibility of photoperiod may be contributing to the proportions of intersex females from winter to summer. When the hours of day light lessened intersex females increased (Dunn et al., 1993) quite possibly at the expense of males. Intersex females may develop during the intermediate hours of daylight (Dunn et al., 1993); thus, when proportions of males, normal females and intersex females were correlated with photoperiod from the sampling latitudes there were significant findings.

In FBC and FBR, sex ratios of all phenotypes (males, normal females and intersex females) were examined for correlations with photoperiod (https://www.esrl.noaa.gov/gmd/grad/solcalc/sunrise.html). As previously mentioned, Dunn et al., (1993) determined intersexes were developed during intermediate hours of daylight. Classifications of daylight are defined as twilite, nautical twilite, and astronomical twilite (Earth System Research Laboratory (ESRL) (https://www.esrl.noaa.gov/gmd/grad/solcalc/. Retrieved 7/4/2018). The phases are defined based upon when the sun is below the horizon during the morning and evening. In FBC, the linear relationships between percentages of males and normal females to photoperiod were mildly correlated but not significant; as daylight hours increased percentage of male's increased and normal females decreased. When FBR males and intersexed females were correlated to photoperiod both were found to be significant; as photoperiod increased the percentage of males increased and the percentage of intersexed females decreased. The result for FBR (males and intersex females) and FBC (males, normal

females and intersex females) may reveal that the populations are cued by the environmental sex determinate photoperiod and the intersex populations are cued to intermediate photoperiods as reported by Dunn et al. (1993, 2005).

Populations of amphipods with 100% intersexes is extremely rare, if even found in any other population. The evidence that intersexes do occur in a variety of habitats and geographical locations is relatively low; therefore, FBR and FBC are commonly cited as an exceptional case to lower intersex frequencies found in other populations (Ford and Glazier, 2008; Glazier et al., 2012; Miller, 1977, Buikema, 1980; and Martins et al., 2009).

The FBR population (n = 1348) was male biased with a total of 61% male and 39% intersexed females over the entire study; normal females were never observed. The female population peaked during the winter months (Nov – Feb). The per cent of males varied from 55 – 71% of the total and matured males peaked during the fall months. As with the FBC population, FBR showed similar trends between male and intersexed female lolls and peaks; when males were at their highest numbers, females were lowest and vice versa. Causes for this trend may also be under the influence of an environmental parameter (e.g. photoperiod) (Dunn et al., 1993) to date, there is no known feminizing parasite present to explain the 100% intersex condition (further discussed in Chapter 4).

Sexually reproductive male's and female's usually, at least within the same populations, develop similar life cycles and reproductive patterns; theories and models on sexual reproduction predict that a population will maximize its reproduction potential by presenting sexually matured males and females at the same opportune time/season and with a 1:1 (M : F) sex ratio (Charnov, 1982; Fisher, 1930). The populations of FBC and FBR, as described, do not support Charnovs' sex allocation theory and/or the Fisher principal. According to Charnovs' (1982) sex allocation theory, in gonochorisitic (male and female) animals the allocation decision lies between producing males or females and Fisher's principal predicts an overall 1:1 sex ratio. Fisher's principal is based upon the idea that there are equal costs for parents to either produce a male or a female offspring and that populations trend towards a 1: 1 (M : F) sex ratio. If there were more females in a population than

males, a male would have more potential to mate than the female, and parents who invest more in producing males would subsequently have a fitness advantage over parents who produced females (Fisher, 1930). Fisher's principle, however, does not always hold true among circumstances of ratio sex biases (River and Willard, 1973), and there are two situations in which this exception applies, one, within individuals, and two in populations (River and Willard, 1973). At the individual level, a pair of parents may just produce more of one sex such as, more males than females or vice versa, while within the population level, the occurrence of one sex is simply more frequent over all. If there are external factors affecting the ratio (e.g. ESD, parasite, and pollution) it can have profound effects on the population as a whole; in addition, the effects on sex can occur at different stages of development (i.e. primary, secondary and tertiary). During primary development, occurrences during meiosis, chemicals, and hormones can all have an effect on ratio (River and Willard, 1973); secondary development begins at birth and can be affected by temperature (e.g. reptiles), photoperiod (amphipods) and if the environmental cue is persistent it can produce all one sex (River and Willard, 1973); lastly, tertiary is measured at adulthood and involves circumstances such as, mortality, predation and other pressures of selection (River and Willard, 1973). Rivers and Willard (1973) also proposed a model to test the manipulation (in mammals) and the response to environmental stressors. The assumptions were that males in good condition will produce more males, and females in good condition will produce fewer females; however, in harsh conditions and/or in the presence of stressors, females tend to out produce males with more females. This reproductive strategy is to ensure reproductive success (i.e. one male per numerous females). The populations in FBC and FBR appear to be responding to a genetic anomaly (i.e. fixed gene, mutation), an unknown feminizing parasite, or an environmental stressor. Applying the Rivers' and Willard (1973) model, perhaps the populations of FBC and FBR are male biased because males are out producing the females with males and females are producing less females, resulting in more males at the individual level *and* the population level.

FBC and FBR Gammarus minus sexual dimorphism and maturation

Sexual dimorphism is common throughout Amphipoda and based upon the evidence in this study and others, FBC and FBR both adhere to sexual dimorphism; the male being larger than the intersex females and intersex females larger than the normal females (Miller, 1977; Buikema, 1980; Glazier and Ford, 2008; Glazier et al., 2012). Sexual dimorphism is strongly correlated with several functions; one, enabling the males to carry females in moving/flowing waters (Adams and Greenwood, 1983), two, a competitive advantage over other smaller males for females, as well as an advantage to over-taking females (Ward, 1983, 1984), and lastly larger male size enhances the males ability to carry females for longer periods of time, which allows for a competitive advantage over other, usually smaller males (Elwood et al., 1987). Within this investigation, intersex females in both FBC and FBR, were significantly larger than the normal females (in FBC), which is consistent with other studies (Ford et al., 2004; Glazier and Ford, 2008; Glazier et al., 2012). To determine why intersexes are larger than the normal specimens, Sexton (1924) conducted breeding experiments on *G. cheureuxi* and found that all of the intersex males and females each took longer to reach maturity. The energy spent by the intersex animal towards maturation and reproduction was perhaps conserved and then re-directed towards somatic growth, thereby, allowing the animal more time to grow in size before sexually maturing (Ford et al., 2004). Ford et al., (2004), presented several other hypothesis for why *E. marinus* intersexes may be larger than the normal individuals of the same sex, one, a possible correlation based on the degree of intersexuality (e.g. one or two papillae, gnathopod size, etc.) and delayed maturation, two, increased frequency of molts that would increase growth rates, and three individuals possibly displaying intersexuality at later stages of growth. Accordingly, both FBC's and FBR's intersexed female sizes are supported by the studies mentioned above and may be due to a single or combination of the presented hypothesis. The ability of immature intersexes to outcompete smaller females for nutrients could also enhance growth; if the intersex females are under the influence of an unknown (male) hormone, it might be possible for the individual to be stronger and/or more aggressive. This hypothesis would require more research to understand the species endocrine system and hormonal control.

The females' body length patterns for both streams suggest that the breeding cycle for FBR is from late fall to early winter. Based upon the body lengths (hence, sexual maturity) the females would be ready for pre-copulation and mating. Falling Branch Cave females (intersexes) follow similar growth patterns as FBR intersex females; the larger females would be set for engaging in pre-copular and mating, from late fall to early winter, while normal females (based upon growth patterns) would be available year around. The two different growth patterns for females that reach sexual maturity supply sexually mature females continuously year around; hence, the constant mating cycle for FBC.

This study was particularly interested in the traits and characteristics of females with regard to structures and functions, for example concerning body size and how intersex and normal females compare and contrast. When each female phenotype (normal and intersex) mean body lengths were analyzed, all were significantly different from one another, within and among both FBC and FBR. The success of both populations presents a conundrum, because intersexed phenotypes are a negative fitness factor (Ford et al., 2004). Accordingly, Ford et al. (2004), Dunn et al. (1990, 1993), Hatcher and Dunn (1997) all concur that intersexuality causes a multitude of reproductive costs; negative reproductive factors include some of the following, reduced fertility and fecundity, lower embryo survival, reduction in sperm allocation, inability of males to hold or carry the larger intersexed females resulting in excess energy costs, malformations of secondary sexual characteristics (e.g. brood plates and ovaries) and reduced pairing success. The apparent success of both populations (FBC and FBR) is not consistent with the other studies that have defined intersexuality as a negative fitness factor.

Over the 17 month time span, both FBC and FBR intersex females mean body lengths did not present an obvious pattern, while FBCs' normal females did, suggesting perhaps, that the incidence of intersexes is the result of some fluctuating variable (e.g. an environmental variable, parasitic influence and/or pollutant). The patterns observed between mean body lengths and the sex ratios reveal what was anticipated. When females reach population peaks (% sex ratio of total population) they have also attained sexually matured body lengths ($\geq$ 4.8 mm); the relationship perhaps mirrors a reproductive pattern, within each sub population (FBC normal and intersex females;

FBR intersexed females) (Miller, 1977). Accordingly, the adaptation of body length with phenotype (i.e. males larger than females) has been shown to correspond in response to reproductive pressures such as, pre-copulate mate guarding (Naylor et al. 1988; Watt and Adams 1994). Reproductive success is dependent upon male and female amphipod sized relationships (Naylor et al., 1988) to be able to engage in mate-guarding (Hatcher and Dunn, 1997).

When all female populations were examined by season, the winter months are positively correlated (between FBR and FBC intersex females) suggesting that they have similar maturation patterns. Accounting for any lag between intersex and normal female maturation is the assumption that intersexes sexually mature later, which is based on the hypothesis that endocrine disruption may cause delayed development (Ford et al., 2004). Any AGH disruption may be due to the various environmental parameters, pollution, parasites, and other xenobiotics. The chemicals that affect the endocrine system may do so by mimicking estrogenic and hormonal biochemical pathways. These pathways are responsible for the synthesis of hormones and steroids, which regulate maturation, sex determination and reproduction. The significant consequence is found in the increased somatic growth of intersexes, hence the reason for the size differences. Moreover, the two female populations' intersex females are larger and outnumbered normal females for all months of the study. Based upon FBC and FBR females' population data, the assumption that normal females are ultimately more successful than intersex females is not supported.

There was little correlation between environmental parameters and mean body lengths of *G. minus,* with the exception of alkalinity, which was correlated with FBC intersex females mean body lengths, but not significantly. Alkalinity measures the ability of a water body to reduce or buffer acidity, by binding hydrogen ions. Crustaceans' uptake and store ions, such as calcium and magnesium, which is subsequently used for the exoskeleton during growth (Zehmer et al., 2002). This relationship is consistent with studies such as Zehmer et al., (2002) who characterized calcium as a limiting factor in the survival of *G. pseudolimnaeus* post-molt growth.

Intersexuality in FBC and FBR

The occurrence of intersexuality is of special interest. The original percentages that were found in the two streams by Miller (1977) on Falling Branch Road were 100% in FBC and 100% in FBR, but a subsequent investigation conducted by Buikema (1980) found the intersexes in FBC to be approximately 60%. Buikema (1980) also maintained that FBR's intersex population was steady at 100%. Based upon the literature discussed, this rate of intersexuality is truly a phenomenal find that is atypical of amphipod populations.

Falling Branch Road and Cave springs have been studied since the mid 1970's (Miller, 1977) and the sex ratios of both populations remain fairly stable (Glazier et al., 2012); in contrast and as previously illustrated by Glazier (1999), other populations of intersex *G. minus* occur infrequently. In the nine springs sampled by Ford and Glazier (2008) only one male (0.01%) and one female (0.01%), of the total 1,707 *G. minus* collected, were intersexed. The additional sample sites surrounding the geographical area of FBC and FBR include Bradley Run (Montgomery County, Virginia), Antietam Creek (Washington County, Maryland) and Cedar Fork Cave (Adams County, Ohio); of the 405 samples collected from the three sites none were found to be intersexed, which is typical for *G. minus*.

Comparisons of three studies for FBC from 1977 to 2013

Utilizing the three studies of FBC (Miller, 1975 – 1977; Haley 1995 – 1997; and this study 2010 – 2013) comparisons of the environmental parameters were pooled during the same months (May, June, Aug., Oct., Dec. and March) that they were monitored, which is an intermittent forty-three year time span, with no environmental sampling between years 1977 to 1995, and 1997 to 2010. The significant differences between the studies (pH, alkalinity and hardness) may be indicative of either an unknown influence altering the stream system or possibly measurement and/or equipment errors by researchers. Regarding what is known about *G. minus* amphipods, and based upon streams populated with *G. minus* in the Pennsylvania USA region (Glazier et al, 1992), the optimum range of pH is 6 – 7.6, thus, significant differences in pH between this study (7.43) and Haley (7.91) may be related to the decreasing trend of intersex

females. However, three sets of data would not be sufficient to provide truly reliable conclusions.

The correlation between decreasing pH and time (1977 – 2013) may be an example of environmental sex determination (ESD) (Bulnheim, 1966; 1978) and sex ratio (Dunn et al., 1993). Environmental sex determination is a sex choice strategy that maximizes fitness by the organism's adjustment to the local conditions (Dunn et al., 1993). Dunn et al. (1993) determined that *G. duebeni* sex determination cue is photoperiod and a greater proportion of male offspring occur with long-day conditions. If populations of *G. duebeni* are female-biased, then it may be surmised that the environmental cue is a short-day photoperiod and that the cue has had no effect on the androgen gland (AG). As defined in the Introduction (Chapter 1), the gender development of Crustacea depends upon the activation of the AG. If the AG is not activated the animal will be female and if the AG is activated the animal will be male (Charniaux-Cotton, 1960). Interestingly, Dunn et al., (1993) also concluded that intersexuality within the studied population (*G. duebeni*) was associated with the trend of intermediate long- and short-day photoperiods.

In this study, the proportions of FBC females displaying intersexuality and stream pH have declined (100% to 60% and 7.9 to 7.43, respectively). The importance of pH in aquatic systems determines solubility and biological availability of nutrients (or contaminants) to aquatic life (Wetzel, 2001). Considering this, perhaps FBC females and the stream pH are related; therefore, I would propose the theory that pH may be an environmental cue for this population, or even a possible influence on an unknown feminizing parasite (discussed in Chapter 4). As for the cause of the decrease in pH, the immediate area of FBR has undergone increased development of housing, alteration of the landscape and business development, including current and historical coal mining (personal observation and communications). The US Geological Survey (Department of Interior) reports that due to the introduction of various contaminants, groundwater pH and hardness can be altered in areas where mining is active or where mines are found abandoned. Of the many contaminants found in industry and mining operations, the following are specifically toxic to aquatic biota and may have reproductive system effects; cadmium and copper; organic contaminants (pesticides, plasticizers, chlorinated solvents, benzo{a}pyrene, and dioxin (http://water.usgs.gov/

edu/groundwater-contaminants. Retrieved 2018). Based on the activities in the Falling Branch Road area, there may be several of these factors simultaneously affecting this area. If this were the case, and hormone mimicking chemicals were infiltrating the streams, then the effects of these chemicals might be altering the AGH, or other hormonal biochemical pathways. The process of feminization of males has been recorded in several species (e.g. birds, crustaceans, and fish) through estrogen mimics and cellular receptors. If estrogen mimics activate the synthesis of estrogenic hormones, feminization of males is likely.

Since these pathways are crucial in the biosynthesis of hormones, effects may occur from maturation, sex determination, reproduction, and ultimately population structure. Alkalinity and pH are interrelated; alkalinity is dependent on the concentrations of bicarbonates, carbonates, and hydroxides, which are derived from limestone bed rock (Chapter 2). The buffering capacity, or the ability of a water body to neutralize acids and bases, is the primary description of alkalinity (Wetzel, 2001), and one of the most important to aquatic biota. A water body with high alkalinity such as in FBC will experience less deviation within its own acidity, even with introduced pollutants (Wetzel, 2001). Over time, all of the environmental parameters in FBC have had changes; some (pH, alkalinity and hardness) significant and others (DO and stream temperatures) slight. Stream temperatures have slightly increased over the 43 year time span and DO has decreased; DO is negatively correlated with stream temperatures, as cold water maintains higher DO than warm water (Wetzel, 2001). Therefore, it would be logical that DO decreases with the warming stream temperatures, as in FBC. The repercussions of increasing stream temperature and decreasing DO is metabolic stress on aquatic organisms (Glazier, 2009).

This study has given insight into the populations of Falling Branch Cave and Falling Branch Road springs over an extended continuum that has supported previous investigations of both populations (Miller, 1977; Buikema, 1980; Haley, 1997; Ford and Glazier, 2008; Glazier et al., 2012). Within FBC, it was confirmed that the *G. minus* population is male biased, maintains a steady sex ratio between normal and intersexed females (1:3) and maintains a non-seasonal maturation and reproductive patterns. In FBR, the population was also male biased with 100% of females

intersexed; differing from FBC, there was observed seasonal variability of maturation and reproductive patterns.

Environmental Parameters for FBC and FBR G. minus

Amphipods occur in a variety of habitats that include, but are not limited to arctic waters, surface and underground springs, and other marine and freshwaters. Gammaridae amphipods are most successful in thermally stable, alkaline, calcium-rich freshwater springs, spring runs and subterranean habitats (Glazier, 2009). The ionic richness and physical and chemical stability of FBC and FBR habitats are important components to the specific conditions within each spring; while their environmental profiles are significantly different, both populations of *G. minus* appear to have successfully acclimated. Glazier (1999, 2009), states that amphipods are sensitive to fluctuations within their environments and that population densities may decrease with instability.

All of the Falling Branch Cave and Road springs environmental parameters (pH, DO, temperatures, alkalinity and hardness) were stable over the time frame of this study (2010 – 2013). Based upon the ph, alkalinity and hardness of both streams, both of the sample sites may be defined as lotic, alkaline, hard-water spring runs (Glazier, 1990). So, while both FBC and FBR streams are defined equally as alkaline hard waters, they remain distinct.

As previously mentioned Gammarus require specific water chemistry to survive (Glazier, 1990); therefore, significant differences between FBC and FBR environmental parameters may help to define the two separate populations. The distinct difference between FBC and FBR are the location of the streams source waters, with relationship to the actual sample sites. In FBC, the sample site is within 10 – 12 meters of the source (a small cave) and in FBR the sample site is approximately ¼ of a kilometer from the subterranean source (both further discussed in Chapter 2). The individual distances for the two streams have a significant bearing on the parameters of each due to interaction with atmospheric conditions; mean temperatures show less fluctuation over time in FBC, dissolved oxygen is higher in FBR because of the extended time that atmospheric oxygen has to diffuse into the system (Wetzel, 2001). The pH, alkalinity and hardness are all related, but function

as individual constituents within the physical-chemical aquatic system (Wetzel, 2001). The pH is a description of the acidity of a water body. Several ways that pH can be altered include pollutants, acid rain and diffusion of carbon dioxide (CO_2); when carbon dioxide (CO_2) diffuses into water and forms carbonic acid it then reacts with calcium and magnesium to form bicarbonates (Bialkowski, 2006). As water flows it also releases CO_2 that subsequently decreases carbonic acid formation, which then increases the pH (Bialkowski, 2006). As described in chapter 2, dolomite and limestone are the bedrock of both sample sites, which are also two formations that can buffer pH changes (Bialkowski, 2006). Maintaining a stable pH requires calcium in the form of mineral salts (i.e. limestone). An increase in pH causes calcium and magnesium carbonates, (i.e. marl) to precipitate out; this will reduce alkalinity and hardness. In FBC and FBR, both alkalinity (315.86 ±9.0 and 222.47 ±12.7490, respectively) and hardness (353.7 ±19.4 and 234.94, ±22.32, respectively), function to keep pH stabilized (7.43 ±0.24 and 7.67, ±0.19, respectively); however, given that pH in FBR is significantly higher than the pH in FBC, it may give reason to why alkalinity and hardness are lower in FBR.

A low alkalinity decreases the buffering capacity to stabilize the pH; so, due to the buffering capacity of FBC, the pH is lower (Wetzel, 2001). The differences in pH, alkalinity and hardness between the two streams would be partially a function of the length of time the streams are exposed to the atmosphere, prior to reaching the sample sites. Wetzel (2001) also states other factors effecting water bodies could be any of the following; pollution, run off, and atmospheric conditions.

The difference between sex ratios in FBR (100% intersex females) and FBC (60 – 75% intersex females) may be indirectly due to the higher pH levels in FBR. Variable pH in an aquatic system has been shown to increase/decrease the bioavailability of chemicals of interest (i.e. personal care products and pharmaceuticals) (Rowett et al., 2016; Lui and Wong, 2013). For example, pharmaceuticals and other personal care products (PPCR) have become of great interest and concern because of the issues of human health and estrogenic affects (Colburn et al., 1997) and as such, these chemicals have come under increased scrutiny (Lui and Wong, 2013). Triclosan, a commonly utilized antimicrobial agent, is one such chemical. This chemical is in many consumer products (i.e. cleaning products) and more than 85% in an estimated

140 personal care products (European commission, 2009). To determine fluctuations in bioavailability based on pH, an ecotoxicology investigation conducted by Rowett et al., (2016) utilized *G. pulex* to test the affect of triclosan toxicity. Since most laboratory test use "synthetic waters" Rowett et al., (2016) mimicked the freshwaters where the amphipods were collected by including humic acid, sewage effluent (activated sludge) and dissolved organic carbon (DOC), to the aquatic test environment. To evaluate the pH effect, amphipods were exposed to different concentrations of triclosan at pH 7.3 and 8.4, over a series of several tests. Holding all other variables stable, the endpoint (immobilization of *G. pulex*) for this exposure was the measure of effective concentration (EC). The results from the study determined that pH can have a significant effect on the toxicity of triclosan to *G. pulex* via fluctuations in bioavailability when pH is altered. This example (Rowett et al., 2016) illustrates how environmental parameters can affect bioavailability of organic chemicals. Considering that both the pH and proportion of intersexed females are higher in FBR than in FBC, there may be an unidentified chemical (i.e. endocrine disrupter) in FBR that is bio-available in more alkaline waters. To determine this, further study is needed to deduce the quality of surface waters in the area, as well as, ground water tracing to determine the source of recharging zones, origin of streams and convergent streams.

Based on personal observation that FBC is always more densely populated than FBR, I would propose that FBC is a more favorable and stable environment for the animals than FBR. The higher density of animals in FBC is probably due, in part; to higher alkalinity, hardness and concentrations of Ca^{2+} and Mg^{2+} that provide more ion availability to amphipods (Glazier, 1992). Overall, the pH of both streams is within the optimum range (6.0 – 7.7) for most Gammarus (Glazier et al., 1992). Atmospheric conditions also affect dissolved oxygen saturation in lotic streams (Wetzel, 2001).

The interaction with oxygen and surface water increases the DO via diffusion of free molecular O_2 (Katznelson, 2004), thus the more time the stream is exposed to the atmosphere, the higher the DO may become. Falling Branch Cave DO is significantly lower than FBR DO, in part, as waters emerge directly from subterranean sources the DO is initially lower (Wetzel, 2001; Reid, 1961) and the atmospheric exposure time.

Both FBC and FBR are characterized as lotic, but due to the topography, FBR is subject to runoff that occasionally leads to higher stream depths and unknown influents (Miller, 1977). In FBR, these increases can lead to changes in flow rates, dilution of ions and even the density of organisms, as the organisms may be washed downstream. Miller (1977) determined that population density was much lower in FBR than FBC, which was contributed to rising water levels and larger substrate particle sizes. Personal observation during rainfall events in the FBR area, confirm that the stream has, on occasion, risen and increased water flow from receiving runoff and other influents.

The present investigation into the population structure of both, FBC and FBR has provided more insight to the present knowledge of these amphipod populations. The following summarized points are new items for consideration as to the causes of intersexuality, and a possible source of the decreased sex ratio (i.e. intersexed females' decline of 35 – 40% since 1977) within FBC, as well as, trends between FBC and FBR.

- The percentage of intersexed females in FBC has significantly decreased from 1977 to 2013 by approximately 35 – 40%. The decrease in pH and intersexed females may be interrelated.

- Maturation patterns were correlated between FBR and FBC intersexed females; both, were larger in winter than in any other season.

- FBC intersexed females were positively correlated with stream temperatures; as temperatures increase, intersex female ratios increase.

- Photoperiod was significant for FBR males and intersex females; long day - % of males was greater and short day - % of intersex females was greater.

- Photoperiod was significant for FBC intersex females; long day - % intersex females were greater, and males were positively correlated with photoperiod. Normal females were negatively correlated with photoperiod; as day light increased, % of normal females decreased.

Overall, this study has revealed that the prevalence and success of intersexuality is an extraordinary occurrence to be reconsidered in population structure.

4. Causes of Intersexuality and Parasitic Prevalence in *G. minus*

4.1 Introduction

Intersexuality, as defined in the introduction (Chapter 1) refers normally to a gonochoristic organism that possesses both sex characteristics internally, externally or both. The widespread occurrence of intersexuality has been noted and reported in crustaceans, such as crabs, lobsters, mollusks and amphipods (Zou and Fingerman, 2000; Gingsburger-Vogel, 1991; Bulnheim 1965; Sangalang and Jones, 1997). The exact mechanism causing intersexes is not well understood although several potential factors (i.e. genetics, ESDs', and cytoplasmic parasites) have been identified (Bulnheim 1978; Gingsburger-Vogel 1991, Dunn et al. 1996). For this study, vertically transmitted (cytoplasmic) parasites were of interest to hopefully resolve the cause of the highly intersexed female populations in FBC and FBR. Feminizing (cytoplasmic) parasites have been shown to influence sex-ratio distortion, and are often referred to as reproductive parasites (Ginsburger-Voel and Desportes 1979; Dunn et al., 2001; Terry et al., 2004; Werren et al., 2008; Engelstaedter and Hurst, 2009; Cordaux et al., 2001; and Short et al., 2012).

If the populations of this study were influenced by feminizing parasites, it would go a long way in resolving why the population's female intersexuality is so high. Some host-parasitic relationships that exist in aquatic and terrestrial crustaceans include 1) *Wolbachia* bacteria and isopods 2) Microsporidia and amphipods (Bouchon et al., 1998; Riguad and Juchault, 1998; and Terry et al., 2004) and 3) Paramyxea and amphipods (Short et al., 2012). Evidence supports a strong positive correlation between parasitic prevalence and the host response of intersex development for numerous crustaceans in natural populations; however this does not confirm definitive cause-and-effect (Dunn and Smith, 1993, 1996, 2001; Dunn et al., 1995; Kelly et al., 2004). Cause–and–effect has been shown in controlled experiments that manipulate and/or isolate sex determining factors (i.e. photoperiod, temperature, androgenic gland ablation, and parasitic influence) (Dunn et al., 2005; Charniaux-Cotton, 1954). It was anticipated that the cause of the male biased sex-ratios and highly intersexed female populations in both FBC and FBR, would perhaps be under parasitic influence.

Thus, the presence and high prevalence of a parasite known to feminize males to either, genetic or phenotypic females may be a conceivable cause (Jahnke et al., 2012, Kageyama, et al., 2012). Based upon this evidence, the amphipods were investigated for the presence and prevalence of *Wolbachia* bacteria, Microsporidia and Paramyxea parasites.

Wolbachia are obligate endosymbiotic bacteria that belong to α – Proteobacteria (O'Neill et al., 1992; Rousset et al., 1992) and are known to infect terrestrial isopods and other crustaceans (Cordaux et al., 2012). The terrestrial isopod *Armadillidium vulgare* (order Isopoda, suborder Oniscidea; classification from Martin and Davis, 2001) has been well studied and known to be feminized by *Wolbachia* sp. (Roussett et al., 1992). Feminization in this instance, is suggested to occur due to a cytoplasmic sex determinate (CSD) modulating the endocrine control of sexual differentiation (Rigaud, 1997), in which genetic males are converted to phenotypic females. In addition, some populations of *A. vulgare* host a bacterium of the genus *Wolbachia* that maintain a symbiotic relationship (Rousset et al., 1992). Through this symbiosis, a peculiar mode of sex determination occurs that is typical of CSD: all the organisms in lineages infected by *Wolbachia* have a male genotype of ZZ, and females result from the presence of this bacterium in the cytoplasm of the cells (Rigaud and Juchault, 1992, 1993). Vertical transmission of *Wolbachia* ensures the propagation of the parasite. Most CSD's follow the maternal path and are passed from generation to generation distorting the populations normal sex ratios, for example, the infected A. vulgare females will subsequently produce approximately 90% daughters, which skew the normal 1:1 (male: female) sex ratio within their respective populations (Juchault et al, 1993; Dunn et al., 1995; Terry et al., 2004). Furthermore, as a result of incomplete feminization via CSD of the bacterium, intersex phenotypes can range from an intermediate male and female, to functional females (with male secondary sex characteristics), and fertile intersexes to sterile males (with female genitals) (Rigaud and Juchault, 2011). While *Wolbachia* spp. has been found in 61% of isopods, Cordaux et al., (2012) also revealed non-isopod crustaceans (amphipods and ostracods) that possess *Wolbachia* spp.

Cordaux et al. (2001) have shown that crustacean hosts, including two intertidal amphipod species *Orchestia gamarellus* and *Talorchestia deshayesii*, harbor

Wolbachia strains. *Wolbachia* strains in these amphipods are the first evidence of Crustacea infection outside the order Isopoda. Subsequent to Cordaux et al., (2001), greater numbers of Crustacea were discovered harboring *Wolbachia* that included the following: Class: Malacostraca, Order: Amphipoda and Isopoda; Class: Maxillopoda, Infraclass Cirripedia) (Cordaux et al., 2012). Moreover, *Wolbachia* were also found within these orders; Amphipoda (O) *Talitrus salator, Armadillidium granulatum, Armadillidium pelagicum Armadillidium sulcatum, Cubaris murina,* and *Hemilepistus reaumuri;* and in Isopoda (O), *Platyarthrus hoffmansegghi, Porcellio albinus, Porcellio buddelundi, Porcellio lamellatus, Porcellio variabilis, Porcellionides cingendus*, and *Trachelipus rathkei*, and in Cirripedia (I), *Lepas anatifera*. The discovery of *Wolbachia* in the goose-neck barnacle (*Lepas anatifera*) is significant because the class Maxillopoda is a major crustacean group consisting of more than 15,000 species, and when Cordaux et al., (2012) conducted a phylogenetic analysis (based upon the wsp gene), the Maxillopoda *Wolbachia* strain was closely related to other crustacean *Wolbachia* strains (Cordaux et al., 2012). Cordaux et al., (2012) concluded that this closely related strain implicated that *Wolbachia* may adapt to a large range of crustacean hosts. Based upon the findings of these studies, I hypothesized that *Wolbachia* may be present in the studied population of FBC and FBR, which may be the cause of the intersexed condition.

Protozoan parasites in the phylum Paramyxea most notably cause sexual dysfunction and mass mortalities in bivalves of Europe (Boyer et al., 2013) (Villablba et al., 1993). Paramyxea infections produce female-biased broods, as well as intersexuality, as seen in *O. gamarellus* (Ginsburger-Vogel, 1991, GinsburgerVogel and Desportes, 1979). Paramyxea are classified into four separate genera: *Marteilia, Paramarteilia, Marteilioides*, and *Paramyxa*, according to their life cycles (Feist et al., 2009). *Marteilioides chungmuensis* (Paramyxea) is an example of how the parasite affects the Pacific oyster (*Crassostrea gigas*). Ovary enlargement disease affects the commercial end of the demand for oysters, because the enlargement of the ovary produces a less than esthetic appeal to consumers (Itoh et al., 2004). These protists are well studied in bivalves and while different species have individual ways of infecting the organisms, one process they have in common is their cellular development. As Itoh et al., (2004) described, the development cycle of *M. chungmuensis* within the Pacific oyster occurs in five phases: (1) invasion (2)

extrasporogony, where the cells develop an internal cleavage (3) secondary cells are released to the gonad where multiplication occurs (4) sporulation then occurs in the oocyte from the secondary (stem) cells (5) mature parasites are then released from the oyster via the genital canal. The parasite is not well studied in Amphipoda, but what is known is it has the potential to manipulate sex determining mechanisms within *E. marinus* (Short et al., 2012).

In the study of *E. marinus*, it was discovered that not only was the amphipod infected with the feminizing Microsporidian parasite *Dictyocoela duebenum*, but every organism tested was co-infected with an unknown Paramyxea (Short et al., 2012). The studied populations of *E. marinus* presented both female bias and high levels of intersexuality and further screening revealed the genus of *Marteilia* (Paramyxea) parasite (Short et al., 2012). Based on these findings Short et al., (2012), proposed the hypothesis that perhaps the actual feminizing agent is not Microsporidia, but is a feminizing Paramyxea. The two (Microsporidian and Paramyxea) which have co-evolved, can then utilize the same pathway to infect *E. marinus*, also referred to as a hitch-hitching strategy (Short et al., 2012). The proposed hypothesis by Short et al., (2012) that a Paramyxea and not Microsporidia was feminizing the studied population of *E. marinus* was further supported, albeit not in the *E. marinus* population, but by the findings of the investigation, by Pickup and Ironside (2017), on beach-hoppers. The amphipod crustaceans *Orchestia gammarellus* and *O. aestuarensis* (beach-hoppers) are frequently co-infected by two feminizing parasites {Paramyxea (*Paramarteilia orchestiae*) and Microsporidium (*Dictyocoela cavimanum*}, which give rise to female–only broods (thelygenic) along with intersexed individuals (Pickup and Ironside, 2017). To determine which of the cohabitating parasites cause the all female broods and intersexuality Pickup and Ironside (2017) conducted breeding studies with infected and noninfected specimens. Several conclusions were drawn by Pickup and Ironside (2017) to include the following, *O. gammarellus* infected with *P. orchestiae* produced significantly higher thelygenic broods and intersexes even without co-habitation of *D. cavimanum*, there was no difference in mortality between the infected and noninfected indicating Paramyxea feminizes in these *Orchestiae* populations. It was also stated, by the study, that the Paramyxea species found in *E. marinus* was not the same as that of *Orchestiae* populations, thus an indication that there could be a large divergence of Paramyxea within crustaceans,

as well as, a variety of feminizing mechanisms. The outcome of both studies (Short et al., 2012 and Pickup and Ironside, 2017) gives promise to investigations into the intersexed condition of FBC and FBR and the possibility of Paramyxea influence.

In Amphipoda, the most notable host-parasitic relationship includes amphipods infected by Microsporidia. There are approximately 187 genera and more than 1300 species of Microsporidia, among these about half infect aquatic species, and approximately 50 genera infect aquatic arthropods (Madyarova et al., 2015). Microsporidia are ancient eukaryotic intracellular fungi, which are either horizontally or vertically transmitted (Dunn et al., 1995). As described previously, the horizontal transmission is a design that infects different hosts of the same or different species, whereas vertical transmission (transovarial) involve parent to offspring infection (Dunn et al., 1995). Terry et al. (2004) reported that within 16 amphipod hosts examined 11 species of Microsporidia were detected, there were more female amphipods than males infected, which subsequently suggest that host sex-ratio distortion occurred. Based on this distortion, Terry et al. (2004) was able to hypothesize that Microsporidia are transovarial parasites with two transmission pathways. Transovarial is most important to this study.

Little is known about the specific mechanisms underlying how Microsporidia affect their hosts (Stentiford et al., 2013). The knowledge of some extracellular Microsporidia proteins have been elucidated, but specific tools for genetic study of intracellular Microsporidia still face challenges and therefore, much is still unknown about how Microsporidia proteins interact with their hosts. To increase the likelihood of transovarial transmission some Microsporidia parasites are capable of feminizing male amphipods in order to distort the host's sex ratio to favor females (Dunn et al. 1996). Although the exact mechanism is not well understood, the resulting effect is feminization of males. An overall sex ratio favoring females perpetuates the infection ensuring the propagation of the parasite (Keller et al. 1965; Dunn et al. 1996).
Sex determinates such as parasitic influences, directly affect the development of the androgenic gland (AG) that is located at the terminal end of the vas deferens (segment 7). Cytoplasmic sex determinates are believed to disrupt sex differentiation by inhibiting maturation of the AG; the AG secretes an androgenic gland hormone (AGH) that the organisms require to develop the primary and secondary sex

characteristics (testis, vas deferens, spermatozoa, papillae and large 2nd gnathopod with claw) of males (Charniaux-Cotton, 1954). Male sexual differentiation is controlled primarily by the AGH (Charniaux-Cotton, 1954); if the AG is not activated amphipods will remain female and develop the appropriate primary and secondary sex characteristics of females (ovum, oocyte, oviduct, and brood plates) not males.

Microsporidia genera are wide spread within a variety of amphipod species (Ginsburger-Vogel 1991; Dunn et al. 1993; Mautner et al. 2007; Steniford et al. 2013) several known host-parasitic (amphipod – Microsporidia) examples follow;

- *G. duebeni duebeni – Dictyocoela duebenum,* (Dunn et al., 1993)
- *Pallasea cancellus* – Microsporidian *sp*., (Mautner et al., 2007)
- *G. chevreuxi* – Microsporidian *sp*., (Terry et al., 2004)
- *Eulimnogammarus marituji – Dictyocoela sp*., (Madyarova et al., 2015)

To the best of my knowledge, *G. minus* from FBC and FBR, Virginia, USA populations are not known to harbour feminizing parasites (Buikema, 1980; Ford and Glazier, 2008). The results for both studies utilizing histology methods were negative.

In chapter three, we established that there are exceptionally high occurrences of female intersexuality and male biases within both FBC and FBR populations. Therefore, molecular and histological methods were utilized to determine if either population was infected with any of the known feminizing parasites. The identification of any newly found parasites would then be sequenced for possible identification.

The aim of this component of my thesis was to determine whether intersex specimens are more likely than normal specimens to be infected with parasites known to induce feminization and/or intersexuality.

4.2 Materials and methods

4.2.1 Amphipod sampling

To detect and assess parasite prevalence, *G. minus* were collected from Sep 2011 to Sep 2013 from the two sites described in Chapter 2 (Falling Branch Road and Falling Branch Cave Springs). Amphipods were collected via standard kick sampling methods (see Chapter 3). Animals representing each phenotype (FBC 80 males, 80 normal females, 80 intersex females, and FBR 80 males, and 80 intersex females) from each sample site were utilized for parasitic detection via molecular techniques. The specimens were stored in plastic containers (1 liter) with spring water, placed in a cooler with ice and transported to the laboratory. At the laboratory amphipods were anesthetized in carbonated water (spring water with CO_2 pellets), stored in 70% ethanol and then microscopically sexed and separated into males, females and intersexes by sample site. At the start, sex could be determined within each sample site by size (males > 6mm, intersexes > 5mm and females >4 mm) subsequent identification would require viewing each animal with a dissecting microscope to view the secondary sex characteristics. Amphipods phenotypes were ultimately described and categorized as male, normal female and intersex as defined (see Chapter 1).

4.2.2 Histology

Of 100 animals collected (in Sep 2011 from FBC and FBR), and taken to the laboratory, 20 were immediately anesthetized in spring water containing carbon dioxide pellets and sexed for the histology process {FBC Males (4), Normal Females (4), Intersexed Females (4) and in FBR Males (4) and Intersexed Females (4)}. The remaining 80 animals were used for molecular processes (DNA extractions, PCR's, and gel electrophoresis).

Specimens were fixed in 2% formaldehyde and 0.04% glutaraldehyde in spring waters, in 4% formaldehyde in phosphate buffered saline (PBS) solution prepared

with distilled water. Fixed samples subsequently were dehydrated in an ethanol series (15, 30, and 50% EtOH in PBS), and then stored in 70% ethanol.

To provide 0.5µm cross sections at the gonad region, animals were first dissected into 2 – 3mm tissue sections from approximately the 4^{th} to the 8^{th} thoracic segments (Figure 34); further, the thoracic legs were trimmed and the remaining tissues were embedded into paraffin cassettes for further sectioning. The gonad region was sectioned to determine the presence of any feminizing (vertically transmitted) parasites. Once the paraffin was set, each sample was sectioned with a manual microtome, 10 slices of the tissues were subsequently placed on slides and allowed to dry. Slides were stained (Hematoxylin & Eosin Staining, Mayor 1904) utilizing the MMI H&E Staining Kit Plus (PN 70302), according to the manufacturers protocol. The H&E staining kit was utilized due to the simplicity and economic features.

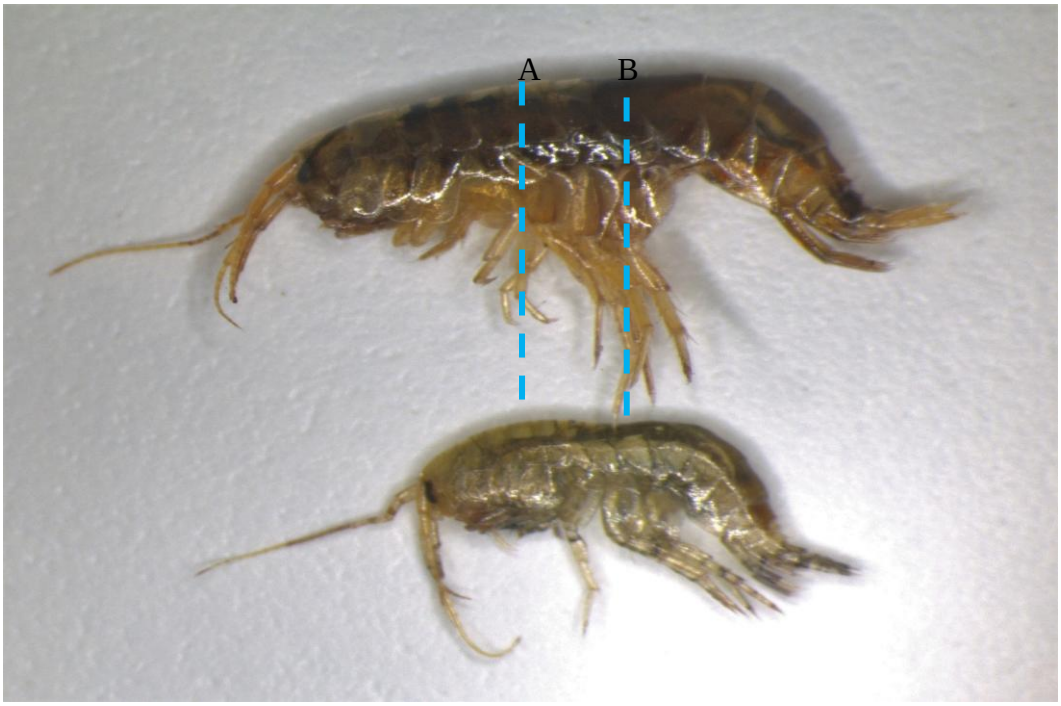

Figure 34. Area of anatomy where *G. minus* were sectioned. The section from the 4th to 8th thoracic segments (marked with blue bars) between the letters (A and B) contains the partial gonads for the amphipod. This dissection measured approximately 2 – 3mm in length. Photo taken by Tamela Brown, 2015.

Amphipod genomic DNA was extracted utilizing the Omega Bio-teck Tissue DNA Spin-Down Method protocol for blood and tissue: DNA breakdown was initiated by placing whole body amphipods and 200 µl of TL Buffer into individual 1.5 ml micro centrifuge tubes and manually ground with a pestle. Twenty-five µl of OB Protease Solution was then added and tubes were vortexed to mix thoroughly. All of the tubes were incubated in a water bath at 55^{o}C for 4 hours until the tissue was completely lysed. After incubation samples were centrifuged at 10,000 (xg) for 5 minutes and the supernatant was transferred to a new 1.5 mL micro centrifuge tube, next 200 µl of BL Buffer was added to each new tube. Tubes were vortexed to mix the buffer thoroughly and incubated at 70oC for 10 minutes, next 220 µl of 100% ethanol was added to each tube. The contents of each micro centrifuge tubes were then transferred into a DNA mini column and 2mL collection tube, which was centrifuged at maximum speed 14,000 (xg) for 1 minute. The filtrate was discarded and the collection tube reused for the next step. Next, 500 µl HBC buffer diluted with 100% isopropanol was added to each tube and centrifuged at 14,000 (xg) for 30 seconds. The filtrate was discarded and the collection tube reused for the next step. Next, 700 µl DNA Wash Buffer diluted with 100% ethanol was added to each tube and centrifuged at 14000 (xg) for 30 seconds, repeated. To dry the columns they were centrifuged another 2 minutes and then each of the DNA mini columns were transferred into new nuclease-free 1.5 mL micro centrifuge tubes. Next, 100 µl of Elution buffer was added and heated to 70oC and then let sit at room temperature for 2 minutes, and then centrifuged at 14000 (xg) for 1 minute, repeated; finally, the eluted DNA samples were stored at -20°C. DNA was quantified by spectrophotometry (Nano Drop). DNA samples were utilized to verify presence or absence of parasitic infections. The parasite groups that were chosen for study were known to infect crustaceans and so were prioritized by their known influence on the feminization of males and/or other population effects (i.e. sex ratio distortion) ((Terry et al., 2004; Short et al., 2012; Yang et al., 2011; Baker et al., 1995; MacNeil et al., 2003). Therefore based on the evidence, parasites were initially targeted by PCR screened based methods, utilizing primer set (18SF, 981R) for non species-specific Microsporidia. Subsequent to a positive PCR product, a species-specific (VIF, 1342AC) primer would follow. The non-species-specific primer had been adopted from previous studies (18sf, Baker et al., 1995; 981r MacNeil et al., 2003) and was expected to give a positive PCR product {890bp fragment, Microsporidia small

subunit of rDNA (SSU rDNA)} for the *G. minus* amphipods in FBC and/or FBR populations. Following a positive PCR reaction for the initial primer, sequencing by the Sanger Method would follow.

Table 16 Parasite identification screenings. Parasite name, primer, primer source, reference, primer sequence and PCR thermal conditions for the parasite identification screenings for the Falling Branch Cave and Road Springs populations of *G. minus*.

Parasite Name	**Primer**	**Primer Source**	**Reference**	**Sequence 5' end to 3'**	**Thermal cycle**
***G. minus* CO1gene**	LCO1490 HCO2198	Folmer et al (1994)	Short et al., 2012	GGTCAACAAAT CATAAAGATAT TGG TAAACTTCAGG GTGACCAAAA AATCA	94°C (5 min), 40 cycles of 94°C (45s), 50°C (45s), and 72°C (45s), a final incubation of 1 min 72°C).
Microsporidia	VIF 1342AC	Weiss et al (1994) Adapted from McClymo n et al (2005)	Short et al., 2012	CACCAGGTTGA TTCTGCCTGAC GGTTACCTTGT TACGACTT	94°C (5 min), 42 cycles of 94°C (45s), 60°C (45s), and 72°C (1.45 min), a final incubation of 5 min 72°C.
Microsporidia	18SF 981R	Baker et al (1994) MacNeil et al (2003)	Short et al., 2012	GTTGATTCTGC CTGACGT TGG TAAGCTGTCCC GCGTTGAGTC	94°C (5 min), 42 cycles of 94°C (45s), 60°C (45s), and 72°C (1.45min), a final incubation of 5 min 72°C).
***Wolbachia* 16S**	Wol6SF Wol16R	Pourali et al (2009)	Rousset et al., 1992	CATACCTATTC GAAGGGATAG AGCTTCGAGTG AAACCAATTC	94°C (5 min), 38 cycles of 94°C (45s), 55°C (45s), and 72°C (45s), a final incubation of 5 min (72°C).
***Paramartelia* 18S Marteilia refringens**	Par 18SF Par 18SR	Short et al (2012)	Short et al., 2012	CTACGGCGATG GCAGGT GGGCGGTGTGT ACAAAG	94°C (5 min), 42 cycles of 94°C (45s), 67°C (45s), and 72°C (45s), a final incubation of 5 min 72°C).

The primer set (V1F, 1342AC) that targets the Microsporidia small subunit rDNA (SSU rDNA) gene, within genus *Dictyocoela* spp., was used to target Microsporidian species. This was based on the presence of *Dictyocoela* spp. in *E. marinus*, *G. duebeni and G. tigrinus* (Yang et al., 2011; Terry et al., 2004); it was anticipated that a positive PCR product may result in *G. minus* amphipods in FBC and/or FBR populations. Subsequent to a positive PCR reaction to the species-specific primer, sequencing by the Sanger Method would follow.

In addition, *Wolbachia*, (Wol16SF, Wol16SR) and Paramyxea (Par18SF, Par18SR) were investigated via PCR based screening methods, as described below (Weiss et al., 1994; Rousset et al., 1992). The basis for the selection of primer sets for the detection of both *Wolbachia* and Paramyxea were determined by previous evidence of sex reversal, male-killing, male feminization and/or sex ratio distortion in terrestrial and aquatic crustaceans (Cordaux et al., 2012; Rigaud et al., 1997; Rigaud and Juchault, 1992; Bouchon et al., 2008; Short et al., 2012) (Table 16).

The CO1 gene is one of the most popular markers for population genetics, as the success of detection is generally high, and may even differentiate species. Additionally, the CO1 gene is a good marker as it is commonly amplified and used for screening parasites.

All PCR's were conducted in 25 µl reactions utilizing manufacturers' protocol. Each 25 µl reaction contained the following: 2.5 mM $MgCl_2$, 0.25 mM each deoxynucleotide, 0.5 mM each primer, 1 unit Taq DNA polymerase, 1 x buffer and 1 µl (10ng) of template DNA. To ensure quality of DNA, the CO1 gene was amplified in each sample. PCR products, negative control (no template control, NTC), a proven positive control, Gel Red, and DNA size marker (1Kb) were all loaded onto 2.0% agarose gels with Tris-Acetate-EDTA (TAE) buffer then run on a Carolina Dual 200 at 70 V for 90 minutes. DNA band sizes were then visualized under UV (NIKON camera and Apple computer system). Conditions for the thermal cycling are detailed in Table 14. All samples were sequenced by The Ohio State University, Molecular, and Cellular Imaging Center (MCIC), and then a BLAST analysis was performed against sequences that are stored in GenBank (NCBI, www.ncbi.nlm.nlh.gov).

4.3 Results

4.3.1 Parasitic Prevalence and Identification

Of the total 20 specimens, the histology slide of an FBC intersex female (Figure 35) appeared to present Microsporidia spores. No spores were present in any of the other slides (FBC males, normal females and FBR males and intersex females) (Table 17).

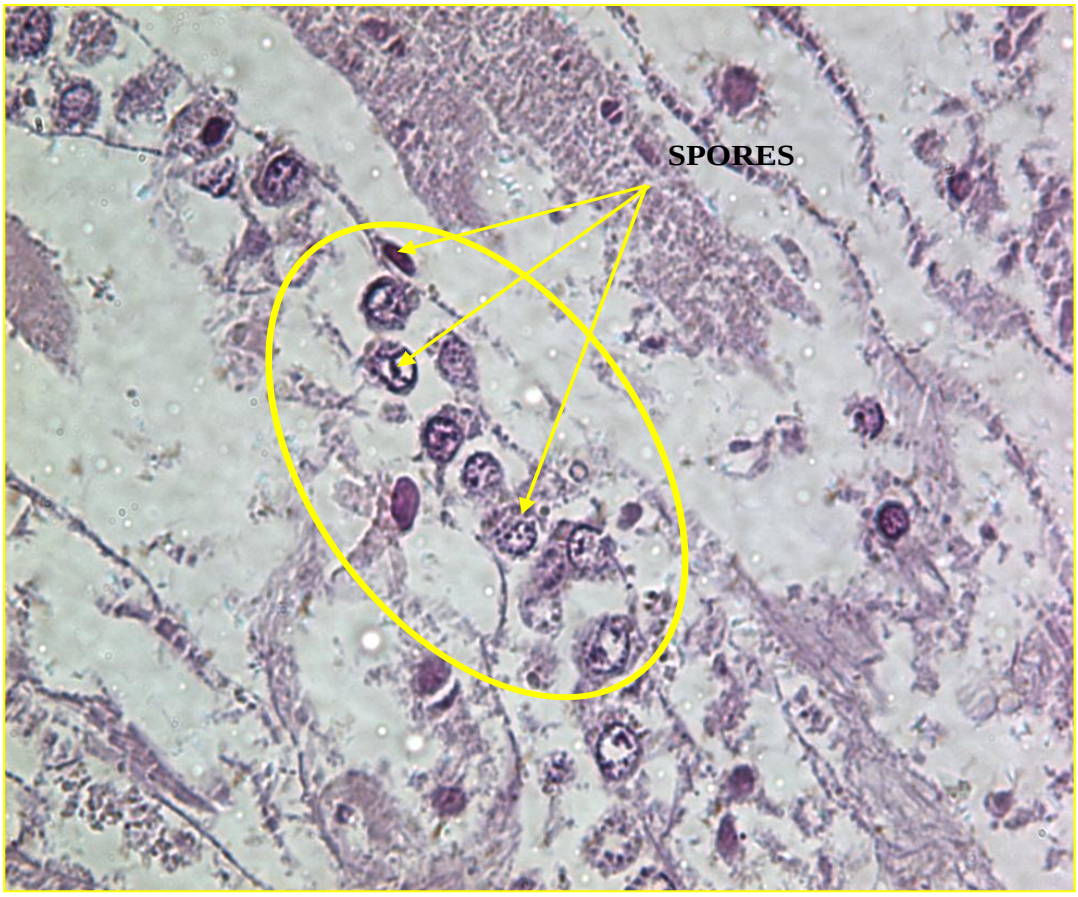

Figure 35. Intersex female (FBR) H & E stained tissue (cross section) of the gonad area of *G. minus*. Within the yellow circle are what is believed to be Microsporidia spores. Magnification 63X's.

Table 17. Table of Results for *G. minus* Histology Results. Four samples of each phenotype for a total of 20 organisms screened. One of four FBC intersex females was positive for possible Microsporidian spores (25%) and one of 20 (5%). Negative results included; 75% of FBC intersex females and 95% of the total 20 specimens. All other specimens (FBC males, normal females and FBR males) were 100% negative for spores.

Site/Phenotype	Infected	Non Infected	Totals
FBC			
Males	0	4	4
Normal Females	0	4	4
Intersex Females	1 (25%)	3 (75%)	4
FBR			
Males	0	4	4
Intersex Females	0	4	4
Totals	1 (5%)	19 (95%)	20

4.3.2 DNA extraction, PCR and Electrophoresis

Of the total 400 amphipods screened for parasitic prevalence, 15 intersexed females (FBC 7 and FBR 8) were found positive for Microsporidia utilizing the universal Microsporidia primers 18SF (Baker et al., 1995), 981R (MacNeil et al., 2003); Microsporidia (V1F, 1342AC), Paramyxea and *Wolbachia* were all negative (Table 18).

The PCR-based screening method revealed positive bands for infected intersexed females; the bands were of medium to high intensity (Figure 37). Primers tested good for all parasitic screenings by producing a positive PCR product with the appropriate bands (Figure 36), as well as the cytochrome c oxidase (CO1) gene, which ensured DNA extraction was successful and of good quality.

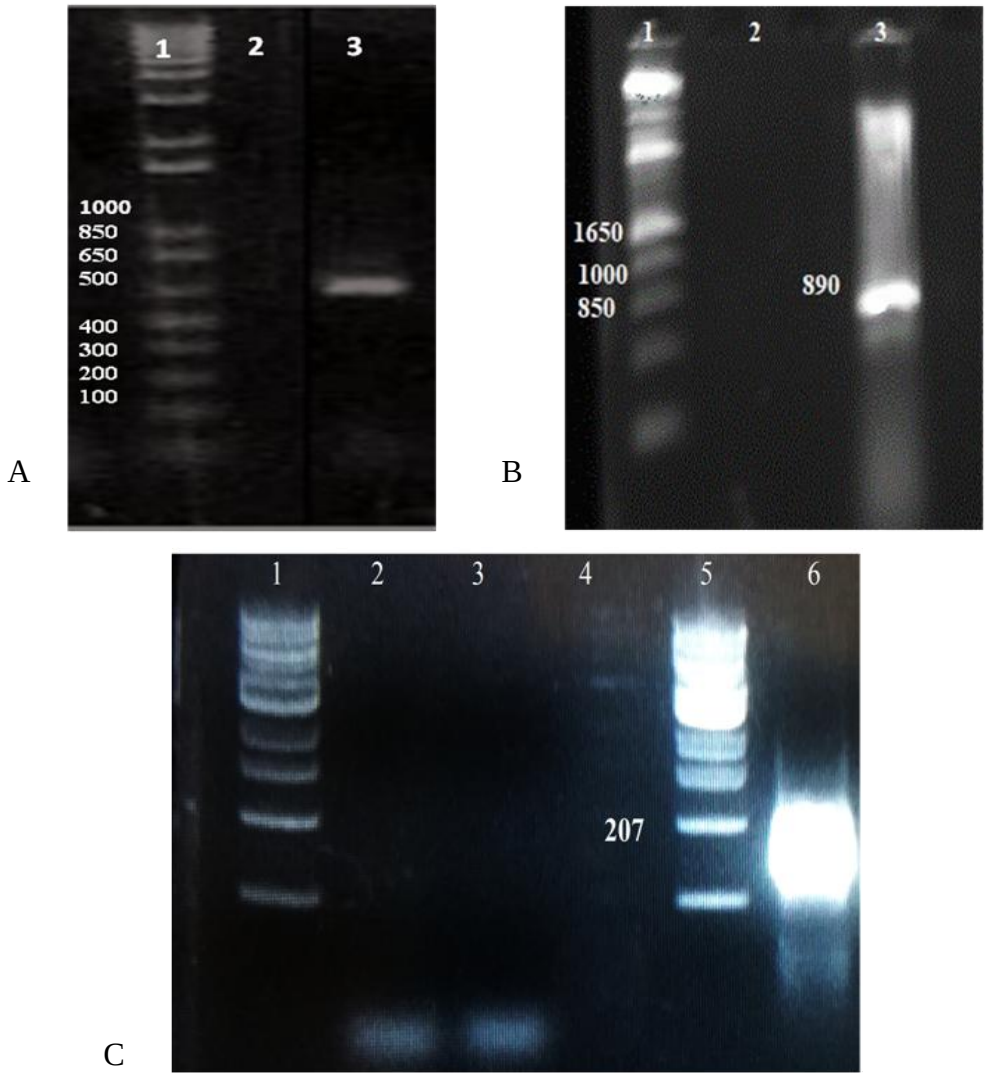

Figure 36. Representative bands of (+ and -). Panel A) *Wolbachia* primer (16S, 500bp) test. Lane 1 (DNA Marker), Lane 2 (NTC), and Lane 3 (PC). Panel B) Microsporidia universal primer (18SF, 981R 890bp) test. Lane 1 (2-Log DNA Marker), Lane 2 (NTC), Lane 3 (PC). C) Paramyxea primer (18S, 207bp) test. Lane 1 (1KB DNA Marker), Lane 2, 3 (FBC and FBR intersex females), Lane 4 (NTC), Lane 5 (DNA Marker) and Lane 6 (PC).

Table 18. Results of parasitic detection and prevalence in *G. minus* amphipods from FBC and FBR Springs. Falling Branch Cave and FBR intersexed females were positive for Microsporidia (non-species-specific primer set 18SF, 981R) at 10% and 11%, respectively. Of the total females screened 9.4% were positive. There were no parasites found for Microsporidia specific primer (V1F, 1342AC), nor for *Wolbachia* and Paramyxea. M (male), nF (normal Female), iF (intersex Female).

Site/ Phenotype	**Microsporidia (18SF)**		**Microsporidia (V1F)**		**Wolbachia (Wol6SF)**		**Paramyxea (18SF)**	
FBC	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected
M	0	80	0	12	0	25	0	40
nF	0	80	0	12	0	25	0	40
iF	7(10%)	73	0	12	0	25	0	40
FBR	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected	Infected	Non-infected
M	0	80	0	12	0	25	0	40
iF	8(11%)	72	0	12	0	25	0	40
Totals	15(9.4%)	385	0	60	0	125	0	200

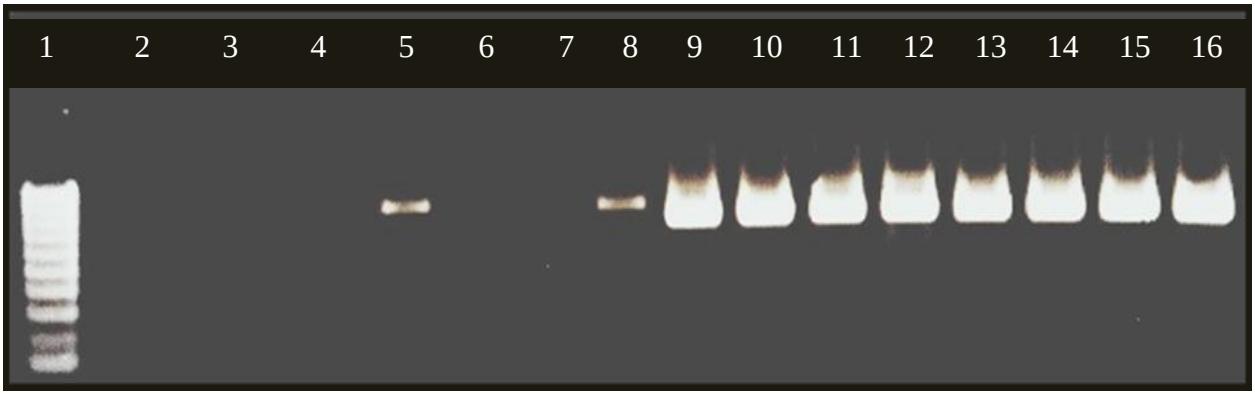

Figure 37. Representative PCR detection of Microsporidia in FBR *G. minus* intersexed females using a non-species-specific primer set (18SF, 981R). Typical examples of bands (+ and -) using the PCR-based parasite screening method. Lane 1 (1 KB DNA marker), Lane 2, 3, 4 (negative for PCR product), Lane 5 (890bp positive for PCR product), Lane 6, 7 (negative for PCR product), Lane 8 (890bp positive for PCR product), Lane 9 through 16 (700bp DNA CO1 *G. minus* gene).

Table 19. Fisher's Exact Test for Infected Proportions versus Uninfected of Microsporidia (18SF) of *G. minus* amphipods collected from Falling Branch Cave and Falling Branch Road Springs. FBC (Falling Branch Cave), iF (intersex female), M (male), nF (normal female), FBR (Falling Branch Road). *Statistical significance @ *p-value 0.05*.

Infected vs Uninfected	**Estimate of difference**	**95% CI for difference**	**Test for difference**	**Fisher's exact test p-value**
FBC iF, FBR iF	-0.0125	(-0.103, 0.078)	Z = -0.27, P-Value = 0.786	1
FBC iF, FBC M	0.0875	(0.256, 0.149)	Z = 2.77, P-Value = 0.006*	0.014
FBC iF, FBC nF	0.0875	(0.256, 0.149)	Z = 2.77, P-Value = 0.006*	0.014
FBR iF, FBR M	0.1	(0.034, 0.166)	Z = 2.98, P-Value = 0.003*	0.007

Within FBC and FBR, proportions of Microsporidia infected intersex females were significantly higher than the other phenotypes (males and normal females) (Table 19). This result is in congruence with what was expected and what has been revealed in other studies (Short et al., 2012; Guler, 2012; Terry et al., 2004) (FBC: Fishers exact test value of 0.014 for both normal females and males; FBR: Fishers exact test value was 0.007, at *p value 0.05*). The Fishers exact test indicated variables were dependent within FBC and FBR (inclusive), but not between FBC and FBR; signifying that variables between phenotypes (FBC and FBR) were exclusive.

.

The PCR product (890bp), for the 15 positive Microsporidian infections in both FBC and FBR intersex females, were sequenced (Sanger method) (Figure 38) and then a BLAST analysis was performed against stored sequences in Gen Bank (NCBI, www.ncbi.nlm.nlh.gov). All 15 of the retrieved Microsporidian (18SF rDNA), were identical to one another, and 97% related to a Microsporidia spp. found in the amphipod *Micruropus wahlii,* an endemic species of the ancient fresh water lake, Lake Baikal, Russia (Madyarova et al., 2015). The parasite species found in *M. wahlii* was identified as *Microsporidia incertae sedis* (NCBI: txid469895) (Madyarova et al., 2015).

All phenotypes for both FBC and FBR were negative for the species-specific microsporidian (V1F, 1342AC), as well as, *Wolbachia* and Paramyxea. The result of the phylogenetic analysis was based on the 16S region of the unknown Microsporidia species found within *G. minus* (Figure 39).

AACATCGATGCTTAACAGTTCATGTCTGTGTAGCAAGGACGA
ACAGCTCACTAGAACTGCGATGATTTACTCTGGCCGGGAGGA
TACCCACGTGAAAATGTGGCTAAGAGGGGGCAGAATAAGAC
GTAGGACTATCAGTTAGTTGGTAGTGTAATGGACTACCAAGA
CGGTAACGGTTGACGGGGAATTAGGGTTCTATGCCGGAGAGG
GAGCCTGAGAGATTGCTCCCACGTCCAAGGACGGCAGCAGGC
GCGAAAATTGCCCACTGTTTGGAGGAGGCAGTTATGAGACGT
GAGAGCGAGTGCTTGGCAAAGAGAAGCAGGAGAATTGGAGG
GCAAGTTTGGTGCCAGCAGCCGCGGTAATACCGACTCCAAGA
GTGTGTATGAGAGATGCTGCAGTTAAAAAGTCCGTAGTCCTG
CTTACGAAACAGGGGGCGACCCCTTCTTGACGAGGTGGAGGA
GCCAATGGGGAGCACAGTATACCAGGGCGAGAGATGAAATG
TCAAGACCCCTGGTGGACTCGGCGAGGCGAAAGCGGTGCTCT
GGTGGGTTTCCGGTGATCAAGGACGAAGGCTGGAGGATCGAA
AGTGATTAGATACCGCAGTAGTTCCAGCAGTAAAAGATGCCG
ACACGCCTGCGGGCAACCGCGGGCGGGGAGAAATCTTAGAGT
TCGGGCTCTGGGGATAGTATGCTCGCAAGAGTGAAAATTAAA
GAAATTGACGGAGCTACACCACAAGGAGTGGATTGTGCGGCT
TAATTTGACTCAACGCGGGACAGCTTACCAA

Figure 38. Sequenced positive PCR product from 15 *G. minus* intersex females in FBC and FBR. The product was amplified by Microsporidian primer (18SF, 981R).

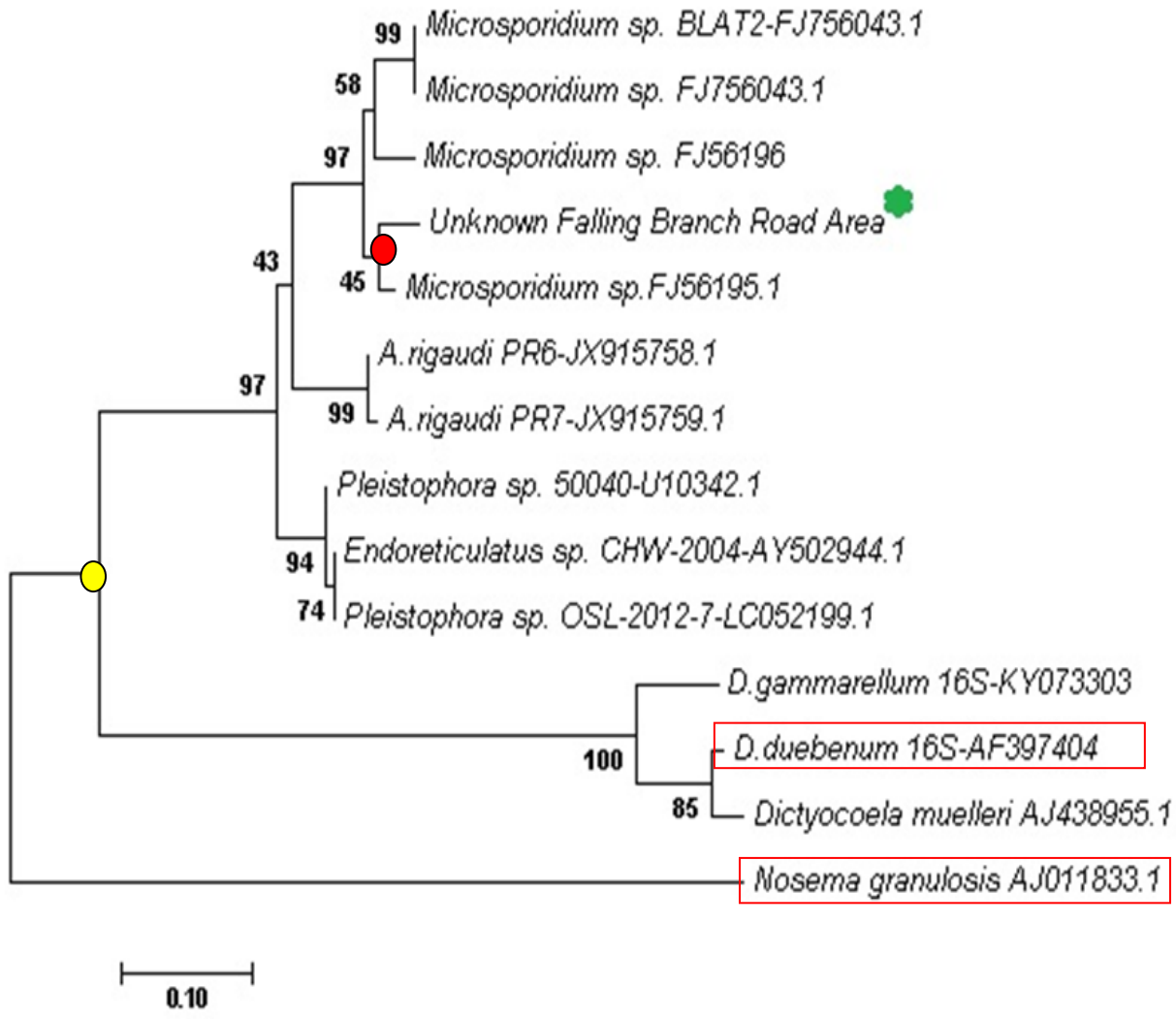

Figure 39. Molecular Phylogenetic analysis by Maximum Likelihood method used to identify the unknown Microsporidia species (green marker) infecting *G. minus* at Falling Branch Road, Montgomery County, Virginia, USA.

A phylogenetic tree was generated using rDNA sequences of parasitic species from NCBI and this study. Sequences were aligned using MUSCLE and a phylogenetic tree was constructed using the maximum likelihood method (Jones et al., 1992) implemented by the MEGA7 program (Kumar et al., 2016). The bootstrap consensus tree inferred from 1000 replicates (Felsentein, 1985) is taken to represent the evolutionary history of the taxa analyzed (Jones et al., 1992). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Jones et al., 1992)]. All branches are drawn to scale as indicated by the scale bar representing a sequence divergence of 10% (Felsentein, 1985). The analysis involved 14 amino acid sequences (Microsporidia small subunit rDNA sequence 16s). All positions containing gaps and missing data were eliminated. There were a total of 168 positions in the final dataset. Description provided by NCBI BLASTn program (Sep. 2018).

The results of the phylogenetic analysis based on the 18S region of the unknown parasite found within *G. minus* intersex females revealed a species or closely related species within the clade of Microsporidian and closest to Microsporidian sp. (FJ756043.1) (see Figure 39). The base pair match to the nearest species of Microsporidian sp. BWOH10 (FJ756195.1) was 97% homologous across the sequenced region. The unknown Microsporidian sp. (green marker) fell within the same clade as Microsporidian sp. BWOH10 (FJ756195.1) and they share the most recent ancestor (red marker). The evolutionary relationship between the two was considered a synapormorphy (based on shared derived characters). The large clade (yellow marker) included the following families (Microsporidea, Nosemadidea, Encephalitozoonidea, Pleisophoridea), which have shared ancestral characters with the unknown species. The Microsporidian sp. BWOH10 lineage includes; Eukaryota; *Opisthokota*: Fungi *incertae sedis*; Microsporidia; *Microsporidian incertae sedis*; and Microsporidia (NCBI; txid698279). The known feminizing Microsporidia of Malacostraca: Amphipoda (red box markers) (*G. duebeni*) within this phylogenetic analysis included, *Nosema granulosis, Dictyocoela deubenum*, and *Octosporea* (genera) (Terry et al., 1998, 1999; Bulnheim and Vavra, 1968). The confidence level for generating this analysis was high. The Maximum Likelihood Method (ML)

utilizes genetic data and probability models to process and propose an unrooted tree of the observed data. This tree is the preferred tree (Swofford et al., 1995).

4.4 Discussion

4.41 Detection and prevalence of parasites

The aim of this study was to detect and determine the prevalence of parasites in the FBC and FBR *G. minus*, as a means of potentially providing evidence for a causal mechanism. If the populations were present for known feminizing, sex reversal and/or sex ratio distorting parasites, then that may be the cause of the intersexed condition. The use of histology and molecular methods were chosen to detect any presence of parasites.

Of the total 460 adult amphipods collected, 20 were preserved and utilized for histology to view the sectioned tissue via microscopic examination. The remaining animals were used in molecular PCR based screening methods for parasitic detection and identification. Histology conducted on the gonad region of 20 specimens resulted in only one FBC intersex female that presented putative Microsporidia spores, which was subsequently verified by the PCR-based screening method. The histology methods were not as revealing as hoped for due to the number of specimens that were negative. However, this result is perhaps not unsurprising as both Buikema (1980) and Ford and Glazier (2008), following their own histological analysis, reported negative findings. With regard to phenotypes: 10% of FBC intersex females were positive, and 0 of 80 normal females and males were positive, and in FBR 11% of intersex females were positive and 0 of 80 males were positive. Thus, the difference in total number of *G. minus* samples for each detection method (PCR's and histology) would have been better served if equal numbers of samples had been processed. As it was, for histology 20 specimens were processed, and for PCR's 200 specimens of *G. minus* were processed

A new Microsporidian species was found in the populations of *G. minus* in FBC and FBR intersex females. Utilizing the universal primer set (18SF, 981R), the novel Microsporidian species was confirmed; fifteen intersexed females (each possessing 2 papillae, brood plates and brood plate setae) were infected with the parasite. This finding partially supports my hypothesis that feminizing parasites would be detected with higher frequency in the intersex females over males and normal females.

Microsporidia were detected and infected versus non-infected was significant, however at such a low occurrence (10 – 11% intersex females versus 0 in normal females) this was not prevalent enough to be considered the cause of intersexuality in FBC and FBR's female populations. Feminizing parasites depend upon the reproductive success of their hosts and since transovarial transmission requires females, it was anticipated that a feminizing parasite would be present in a much higher occurrence. Feminizing parasites are found in males, normal females and intersex females of various Crustacean populations, but the tendency for intersex females to harbor significantly more correlates well with intersexed populations (Dunn et al., 1995; Short et al., 2012, 2015). A female-biased population is a known consequence of feminizing parasites; therefore, even if there were high infections of feminizing parasites the male-biased sex ratio would still not be explained (Dunn et al., 1995; Terry et al., 2004). Of the assumptions put forth, the two remaining were rejected (*Wolbachia* and Paramyxea), as neither were detected within any of the screened specimens.

After a BLAST against known Microsporidia species stored in NCIB, the unknown parasite was found to be 97% related to the Microsporidian species harbored by *Micruropus wahlii*. The amphipod *M. wahlii* is an endemic species of the ancient freshwater lake, Lake Baikal, Russia (Madyarova et al., 2015); the parasite species is identified as *Microsporidia incertae sedis* (NCBI:txid469895) (Madyarova et al., 2015). Utilizing molecular techniques, this study has revealed the first *G. minus* population to be infected with a possible feminizing parasite, and to my knowledge; this is the first study to do so.

Previous studies have revealed intersexes are strongly correlated (if not a result) of feminizing parasites (i.e. *Nosema granulosis, Paramarteilia orchestiae,*) that can induce incomplete feminization (Dunn et al., 1993, 1995; Ginsburger-Vogel, 1991; Kelly et al., 2004; Ironside et al., 2001; Terry et al., 1998; Yang et al., 2011). Knowing how incomplete feminization occurs could perhaps explain the intersexing in both populations. If FBC and FBR were originally all male populations, then it could be that the detected Microsporidia species is responsible for incomplete feminization of males. External intersexuality of male *E. marinus* is associated with the co-infection of *Dictyocoela duebenum* and a Paramyxea protist (Short et al.,

2012). Evidence demonstrates that feminizing parasites can convert their male host into functioning females by manipulation of the androgenic gland, which prevents the differentiation of the male resulting in incomplete conversion (Rogers-Gray et al., 2004; Dunn and Rigaud, 1998; Kelly et al., 2002). Intersexuality is the result of incomplete conversion via naturally, anthropogenic contamination, and/or feminizing parasites (Rogers-Gray et al., 2004; Dunn et al., 2004; Kelly et al., 2002; Kelly et al., 2004). The feminizing parasite hypothesis says that the mode of conversion takes place with two simultaneous events, one feminization and, two demasculinization. Specifically the parasite increases female gene expression and inhibits AG differentiation (Rogers-Gray et al., 2004; Dunn et al., 1998; Kelly et al., 2004).

Short et al., (2012, 2014) have proposed that a "hitch-hiking" strategy involving the co-infection of either two vertically feminizing parasites or one vertically feminizing and one non-feminizing parasite causes intersexuality in *E. marinus*. However, the lack of parasitic detection in high prevalence for both FBC and FBR, leads me to consider alternative causes of intersexuality for these populations and/or possible reasons the feminizing parasite detection failed.

The following issues that may have occurred during molecular processes include, but are not limited to, contamination of isolated amphipod DNA, inappropriate primers, primers that did not work properly, and the use of whole individuals or soft amphipod tissue for the amplification of Microsporidian DNA, as well as overloaded gels. The primers that were used in this study for Microsporidia detection included one non-species-specific universal primer, and one species-specific. It was expected that if Microsporidia were present the universal primer would produce a positive PCR product, as it did (890bp), while the species-specific primer was utilized with the optimism that the positive PCR product would then be identified. The use of whole organisms and/or soft tissues versus haemolymph would not rule out the contamination of the host DNA isolate. Future molecular studies would include an investigation using molecular genetics techniques (SSU rDNA) to detect Microsporidia in the circulatory system (Madyarova et al., 2015). Utilizing the haemolymph of *G. minus* to search for Microsporidia would help to eliminate any detection of Microsporidia found on exoskeleton, the gut lumen, or even inside other parasites of the amphipods. This technique could ensure that the Microsporidia

detected are directly parasitic to the amphipod species (*G. minus*) studied (Madyarvoa et al., 2018). In future studies, a nested PCR would be used to increase the specificity of DNA amplification, accordingly two sets of primer would be used in two successive PCRs. The first reaction of a nested PCR generates DNA products, the products are used in the second PCR reaction (with a second set of primers) whose binding sits are completely or partially different. The final PCR product would be determined by the specificity of the PCR primers. Once primers were selected, new molecular analysis would be conducted.

Further studies would also include breeding experiments to collect data regarding "inheritance" of feminizing parasites (Dunn and Smith, 1993; Dunn et al., 1995). The breeding studies that were attempted for this thesis were not successful, and the issues were difficult to discern. The microcosms that held the collected amphipods were maintained in an environmental chamber, and then as amplexus occurred the pairs were removed and isolated to smaller individual containers. The reason for isolation was to be able to observe when the male released the female in order to note the date of fertilization, as well as to remove the male and place back into the general population.

A noted issue with animal husbandry included 1) the lone female would die within a few weeks. Several causes for this could have been inadequate food sources, water quality and substrate contamination; and 2) when juveniles were released, and the female removed (to prevent cannibalism); immature amphipods would eventually die. Perhaps, the typical monitoring of water quality (pH, temperature, and DO) should be expanded to include all of the initial parameters measured, as well as calcium and magnesium ion content. Research into the husbandry of this species may prove to make a difference. The ability to conduct breeding studies utilizing photoperiod manipulation would certainly be beneficial to verifying the results in Chapter 3, environmental sex determination regarding daylight and sex ratios.

This study has given insight into the Falling Branch Road area highlighting the detection of a novel Microsporidian sp. found within intersex females of both populations. However, Microsporidia was found at such low frequencies it cannot

currently be considered as the cause of female intersexuality in either FBC or FBR. The cause of such high intersex prevalence is still unknown. The following chapter discusses the costs of intersexuality.

5. The costs of intersexuality in *G. minus*

5.1 Introduction

Research indicates intersexuality in crustaceans has resulted in unusual behaviors and substandard reproductive performance. Costs associated with intersexuality may include reduced fecundity (i.e. lower numbers of embryos), reduced fertility (Ford et al., 2003), and the development of secondary sexual characteristics (Ford et al 2003, 2004; Ford and Glazier 2008; Jormalainen 1994 and Ladewig 2002). Although FBC and FBR amphipods exhibit a high prevalence of female intersexuality, they are extraordinary populations because of the success and continued health within both springs. In chapter 3, we observed very high intersexed female populations within both FBC (~ 60 - 70%) and FBR (~ 100%), as well as, substantial population densities (Miller, 1977; Buikema et al., 1980 and Ford and Glazier, 2008; and Glazier et al., 2012). The success of these populations is curious because evolutionary theories would suggest otherwise.

Two theories, which are fundamental to these analyses of normal females versus intersexed females, are sexual allocation (Charnov, 1982; Fisher, 1930) and natural selection (Darwin, 1871). Sexual allocation stresses that the amount of investment given to male and female offspring will be equally distributed and that competition via natural selection for strong, healthy mates will be selected for. Accordingly, a 1:1 sex ratio would be achieved. Charnov (1982) investigated sex allocation theory within unusual reproductive conditions (i.e. hermaphrodites, sex reversals, and intersexes) and using mathematical models predicted that intersexes should not be successful, but should be selected against according to natural selection. FBC and FBR amphipod populations challenge both theories.

Populations with high proportions of intersexuality should not be successful. If a lifetime of reproductive success is less than optimal, then the costs (i.e. densities), incurred, should render a population extinct. Reproductive costs, due to intersexuality, have been shown to be an evolutionary disadvantage (Charnov, 1982).

However, FBC and FBR amphipods are healthy and densely populated (2000 – 10000m^2) (Miller, 1977; Haley, 1997).

5.1.1 Normal and intersexed female fecundity

The body lengths of normal females are positively correlated with the numbers of embryos carried (Ford et al., 2004), while intersex females are not. As body lengths of normal female's increases, the numbers of embryos increase. Ford et al (2003) reported that in the marine amphipod *E. marinus* intersexed females produced ~ 20% less eggs than normal females. Ford et al. (2004) suggested that malformed brood plates (secondary sexual characteristics) may have contributed to the loss of embryos in *E. marinus* or the purposeful maternal ejection of nonviable eggs from the marsupium. However, in another freshwater amphipod species *G. fossarum* intersexed versus normal females, eggs within the marsupium did not significantly decrease during development (Ladewig et al., 2007). Ladewig et al., (2007) determined that the loss of embryos only occurred between developmental stages 5 and 6, which were attributed to successive release of juveniles rather than the intersexed condition.

In 2007 and 2008, 200 – 300 amphipods were collected by Ford and Glazier (2008) from each spring (FBR and FBC) that resulted in three unusual and important findings. One, both spring populations were strongly male biased. Second, the historic intersex condition of females reported by Miller (1977) of *G. minus* was confirmed; the proportions ranged from 73 – 100% intersexed females in both FBC and FBR, respectively. Third, when FBC and FBR were compared to other populations of *G. minus*, the highly intersexed populations were shown to be truly extraordinary; as other populations were very low (≤1%) (Ford and Glazier, 2008).
The specimens from the 2008 study (FBC) were utilized to further investigate fitness and reproductive costs/sexual allocation between intersex and normal females in the FBC population (Glazier et al., 2012). The results of FBC were: 1) intersexed females carried fewer numbers of embryos, but each embryo was larger in mass, than the normal female's embryos, 2) total brood mass did not differ between normal and intersex females, and 3) the results were true for both sampling years and during both

early and late embryonic stages. While all of the results were noteworthy, none were statistically significant and did not affect the overall reproductive fitness of the intersexes when compared with normal females.

For FBC and FBR populations, sex allocation and natural selection predict that due to normal female reproductive success (i.e. greater brood numbers and greater embryonic mass), males would prefer the normal females over the intersex females. These predictions are based on the idea that to perpetuate the species, organisms' strive to produce as many offspring as possible via spreading genes (Dawkins, 1976). Males that select normal females would be ensured that the greatest numbers of embryos and embryonic mass would occur and subsequently ensure reproductive fitness.

5.1.2 Normal and intersexed amphipod behaviors

Various studies have revealed that intersexed amphipods exhibit different behaviors or are the subject of other amphipods behaviors (e.g. males do not allocate sperm equally between normal and intersexed females). The intersexed male *Corophium volutator*, a burrowing amphipod, has been shown to spend more time crawling on the surface than normal males, searching for mates (McCurdy et. al. 2008). McCurdy, et al. (2008) observed the behavior of intersexed male and female amphipods in the laboratory and from a mudflat in Nova Scotia, Canada. At this particular location, predators are absent; which, allowed for simpler observations of mate-searching behaviors. The *C. volutator* male crawls on the surface of the mudflats during ebb tide looking for the females. The females rarely emerge from the constructed U-shaped burrows they have made for themselves; thus, it is up to the male to locate the females and, to engage in pre-copulatory pairing (McCurdy et al., 2008). Since fertilization can take place only after the female amphipods have molted (Sutcliff, 1992) the opportunity for the males to fertilize can be time constrained. The time lost while looking for mates could have an effect on the breeding rates and ultimately the propagation of the species.

Breeding studies by Boyd et al. (2003) have shown that male *G. minus* show no preference for females from different populations and/or different springs. In their study, Boyd et al (2003) crossed males and females from two different populations, which were from two different springs located in Pennsylvania USA. No significant differences in frequency of amplexus resulted in any of the crosses. Since intersexes are not common in *G. minus* (Glazier, 1998) no studies, to date, have specifically determined if *G. minus* males prefer normal females over intersexed females or vice versa. It is well documented that the male initiates the mate-guarding behavior. According to Jormalainen et al (1994), the females are either receptive or will resist male attempts at mate-guarding based upon her reproductive condition. Limitations and other constraints result in vigorous intersexual competition for females, which according to Jormalainen (1994), is most prominent in male biased populations. Mate-guarding and sexual selection have evolved due to the need for favored characteristics (i.e. sexually dimorphic animals) to be passed on to the offspring thus; it is required for the male to engage the female to ward off other males (Conlan, 1991; Jormalainen, 1998).

The female molt triggers the act of fertilization (Culver et al., 1995). While engaged in amplexus the male carries the female with him for approximately two weeks prior to fertilization; the male latches onto the female integument with the second gnathopod and then holds her dorsum to his ventral side (Borowsky, 1984). During this time, the male controls the movement of the pair and continues to isolate her from other males competing for the fecund female. During the mate-guarding process the female does not feed, which takes its toll on the female's fitness. As a result of sexual selection (Glazier, 1999; Wellborn, 2000), studies have determined that male amphipods preferred larger females. Perhaps larger females were better fit and able to withstand the conditions during mate-guarding (i.e. more fat resources) or simply easier to locate.

After the female molts the ova move to the external brood chamber (marsupium) and the male immediately releases packets of sperm into the brood pouch (Culver et al., 1995); external fertilization occurs and the male releases the female. Embryonic development then takes place according to species and water temperatures. In *G.*

minus this is approximately 35 – 40 days and in 4 – 6 stages (Glazier, 1999; Ford et al., 2003).

The primary issues in the populations found in FBC and FBR are that the intersexed condition and the male biased sex ratios challenge various reproductive theories. Many species do have sex allocation ratios of 1:1 and what is perceived to be normal intra/intersexual competition. However, there are numerous examples of deviations that must be explained either by natural selection or evolutionary constraints (i.e. genetic drift, bottlenecking, geographical separation, etc). FBC and FBR are both male biased populations with highly intersexed females, 60 – 75% and 100%, respectively.

In this thesis, the experimental design for mate selection was derived from results of previous studies. Historically, the literature has documented that the intersexed condition has had specific reproductive costs on crustaceans (Ford et al., 2004; Dunn et al., 1990, 1993; Smith and Dunn, 1991). Though there have been significant costs to amphipods in the intersexed condition there have also been minimal costs, as was reported by Glazier et al., (2012) and Ladewig et al., (2007).

In the current study, I tested five hypotheses on behalf of reproductive costs, utilizing a series of experiments. The first two experiments addressed fecundity (e.g. numbers of eggs and embryonic mass) between intersex and normal females, and three specific experiments were conducted to investigate precopular behavior (e.g. male choice for female spring origin, phenotype and body size).

5.2 Aim of the study

5.2.1 Five hypothesis for the two populations (FBC and FBR) of *G. minus*

The aim of this component of my thesis was to determine whether there were reproductive costs to intersexuality in *G. minus*. This was achieved by focusing on the fecundity, size relationships regarding male choice for females, and precopular behavior of normal versus intersex specimens that addressed the following hypotheses:

Hypothesis 1: Intersex females will have significantly fewer numbers of eggs/embryos than normal females.

Hypothesis 2: Males given a choice will show a significant preference for normal females over intersex females (controlling for size).

Hypothesis 3: Males will show a significant preference for females that originate from their own springs (controlling for size).

Hypothesis 4: The average time (seconds) for recently separated pairs to rejoin will be significantly greater for males with intersex females than for males with normal females.

Given the findings reported in chapter 3 that intersex females were significantly larger than normal females, the following hypotheses were also addressed.

Hypothesis 5: Males will show a significant preference for larger females over smaller both in the field collections and laboratory.

5.3 Methods and materials

5.3.1 Experimental Design for Assessing Reproductive Costs of Intersexuality in *G. minus*

To test hypothesis 1, FBC and FBR ovigerous female's embryos were quantified during the entire study from 2010 to 2013.

G. minus were collected (according to Chapter 3 methods) and females were subsequently anesthetized (using carbon dioxide pellets added to spring water). Broods were flushed from the brood pouch with a disposable pipette and counted. Each female length was measured by pushing, the amphipod body and aligning it with a straight edge of a metric ruler. The measurement from the base of the first antenna to the base of the telson was recorded for each in millimeters (mm) (see Chapter 3). Ovigerous female's body sizes (mm) and numbers of embryos were tallied and recorded, for statistical analysis between normal and intersex females.

Hypothesis 2, the experiment was designed to give the males (singletons) a choice between phenotypes (normal versus intersexed females) controlling for size.

G. minus amphipods collected on 22 September 2013, included a total of 75+ amplexed pairs and 25 singleton males from both FBC and FBR each (as described in chapter 3).

Hypothesis 3, males (singletons) were given a choice between females that originated from their own spring or females that originated from another spring (controlling for size).

Hypothesis 4, amplexed males were separated and placed with two different previously amplexed females, one from each spring (FBC and FBR), and timed (seconds) until the male repaired with one of the females. This experiment was conducted on site (Montgomery County, Virginia).

Hypothesis 5, as amphipods were collected the size of amplexed pairs were observed and recorded as a large male/small female pair, or a small male/large female pair.

Within experiment 5, there were two experiments, one, the preferences of a large male for a small/large female, and two, the preferences of a small male for small/large female.

5.3.2 Experiments 1 – 5

Experiment 1 Male choice for phenotypes (controlling for body size)

Fifty amplexed pairs of animals from FBC and FBR each, for a total of 200 amphipods were segregated into separate one liter containers with spring water, leaves and/ or watercress for food, which were then transported back to the lab to conduct the experiments.

At the lab, amplexed pairs from FBC and FBR were segregated according to large and small female body lengths. This was done by sight and was verified by microscopic observations, retrospectively. Each pair was separated by holding in the hand until the male naturally released the female. Each amphipod was placed into a labeled plastic microcosm that contained Deer Park Spring water until there were 20 males and 20 females from FBC and 20 males and 20 females from FBR. Intersexed females from FBR and normal females from FBC were simultaneously introduced into each of the males' microcosms until there was one male + one normal female + one intersexed female. Since FBC had normal and intersexed females, the experiment was designed to ensure that each male was given the proper phenotypic choices (normal and intersexed females). The configuration was conducted as in Figure 40.

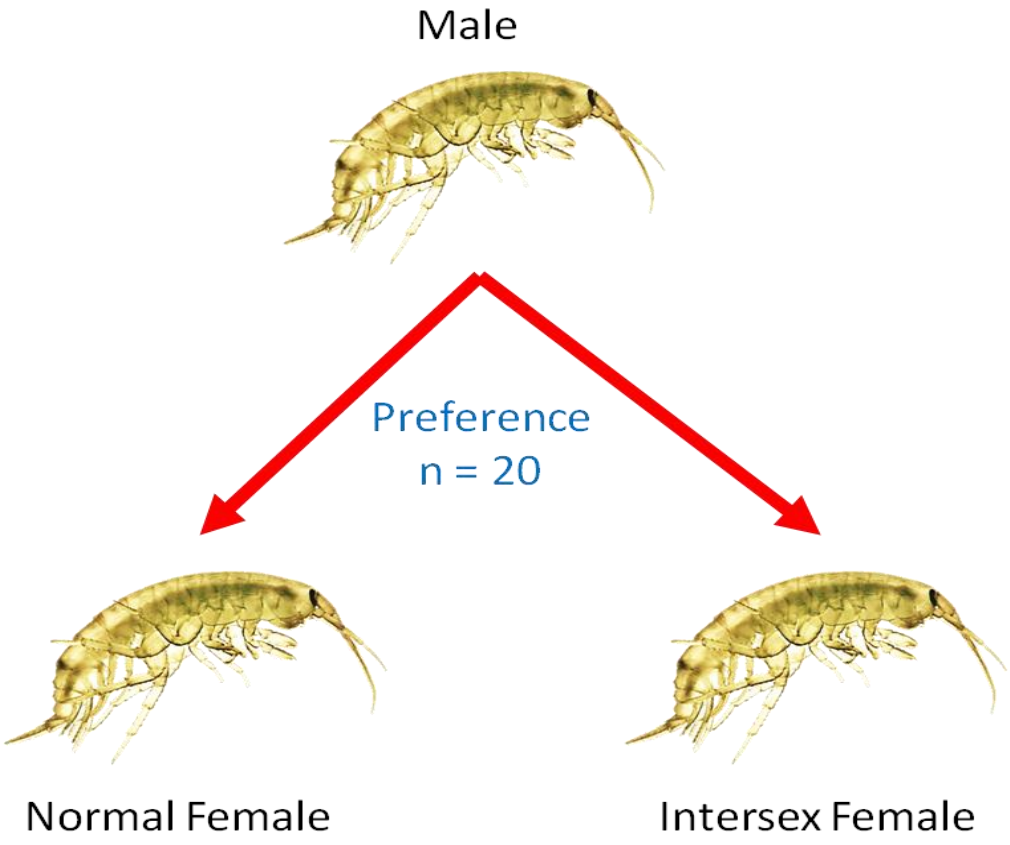

Figure 40. Microcosms for male preference. Each microcosm was 5 cm in diameter, 3.5 cm high, and contained 2 cm (depth) of Deer Park spring water and each contained one male, one normal female and one intersex female for a total of 20 replicates.

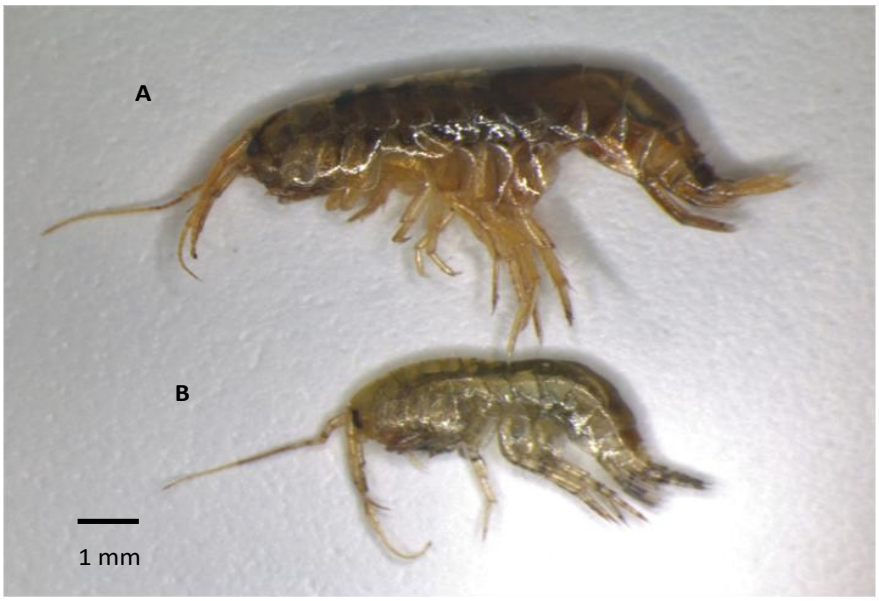

Figure 41. *Gammarus minus* Female Sizes. Large female 6.4 mm (A) and small female 5.2 mm (B). Bar size 1 mm. (Photo by Tamela Brown 2015)

FBC normal females were initially determined by size; as, normal females are noticeably smaller (Figure 41). Due to high mortality associated with using CO_2 to anesthetize the amphipods, therefore all sex determination was verified under the microscope retrospectively. After the amphipods were placed together in each of the microcosms (1 FBC or FBR male + 1 FBC normal female + 1 FBR intersex female) and as soon as amplexus had occurred, the pair was again separated and the female's phenotype determined by microscopic examination and recorded. If the female that was selected to be normal was actually intersexed, the experiment was repeated until a normal female was present with the intersexed male. This configuration only occurred one time. Initially, the third microcosm contained two intersex females; therefore, one was removed and replaced with another female that when examined under the microscope was a normal female.

FBR males were managed in the same manner as the FBC males except that the normal females were from the FBC population. There were only intersexed females in FBR; therefore, utilizing FBC normal females could not be avoided to ensure choice.

Experiment 2 male choices and spring origin (controlling for body size).

Amphipod pairs were separated utilizing the same techniques as in experiment 1. Males from each spring were selected and placed into 40 separately labeled microcosms and then females were introduced. Each of 20 microcosms contained 1 FBC male + 1 FBR female and a second set of 20 microcosms contained 1 FBR male + 1 FBC female. Microcosms were then placed into an environmental chamber at 10^{o}C, no lighting, small bits of dried maple leaves, and left undisturbed for a 24 hour period.

Experiment 3 Re-pairing (Timing)

On 23 September 2013, enough amplexed pairs of animals were collected as described in Chapter 3, from each spring to conduct one trial and 20 replicates for each spring. The experiment took place at the sample site.

The amphipods were gently separated by the same method as in previous experiments and each animal placed into a separate and labeled microcosm (Figure 42). The pairs were reintroduced as follows: the females were placed into the males' microcosm and were timed until they rejoined in the amplexed position. The pairs were reintroduced within ten minutes after each separation to maintain as much continuity as possible. After the pairs amplexed they were placed into separate transport containers.

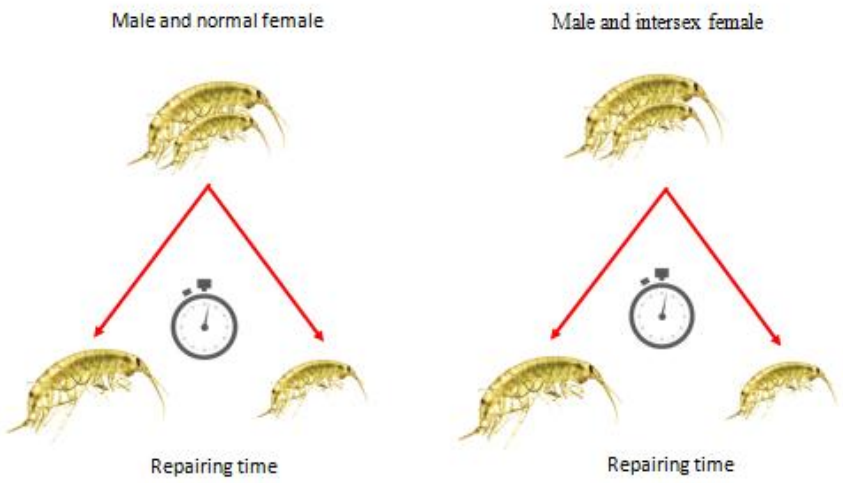

Figure 42. Configuration of experiment 3. The repairing of recently separated amplexed pairs of *G. minus* from FBC and FBR. *G. minus* FBC males and females and FBR in amplexus. Pairs were separated and timed (in seconds) to repair.

Experiment 4 (in the field), large male choice versus female size (smaller female versus larger female).

On March 10, 2018, 20 pairs of amplexed amphipods were collected from each stream (FBC and FBR) according to Chapter 3 methods, for a total of 40 pairs. Forty pairs were observed and recorded as follows; male size and female size of each pair, specifically whether the females were smaller or larger than the males. The pairs were not separated but taken to the laboratory for further experiments on male choice and female size.

Experiment 5 (in the laboratory). Part I. Large male choice versus female size (smaller female versus larger female).

Once at the laboratory, the experiment was initiated; each pair was separated as previously described and the male placed into the microcosm with Deer Park water. Each female was put into a separate microcosm and labeled to ensure pairs were not reunited. After the pairs were all separated and each large male was given two females (one small and one large) the microcosms were placed into the environmental chamber (10^{o}C; no light) with small bits of maple leaves and left undisturbed for a 24 hour period. After the 24 hour period had passed, each microcosm was observed and recorded for pairing.

Experiment 5 (in the laboratory). Part II. Small male choice versus female size (smaller female versus larger female).

Once at the laboratory, the experiment was initiated; each pair was separated as previously described and the male placed into the smaller microcosm with Deer Park water. Each female was put into a separate microcosm and labeled to ensure pairs were not reunited. After the pairs were all separated and each small male was given two females (one small and one large) the microcosms were placed into the environmental chamber (10^{o}C; no light) with small bits of maple leaves and left undisturbed for a 24 hour period. After the 24 hour period had passed, each microcosm was observed and recorded for pairing.

5.2. Results

Hypothesis 1: Embryonic count of normal females and intersexed females.

The mean number of embryos for FBC normal females, and intersex females was 5.5 and 4.2, respectively. The mean number of embryos for FBR intersex females was 4.6 (Figure 43). The ANCOVA revealed that mean embryo counts for FBC intersex females was significantly less than the FBC normal females (ANCOVA, General Linear Model: Minitab 17, df = 2; F = 14.96 p = 0.001 @ *p-value 0.05*). The covariate (mean body length of females), had a slight effect on numbers of embryos but was not significant at *p-value 0.05.*

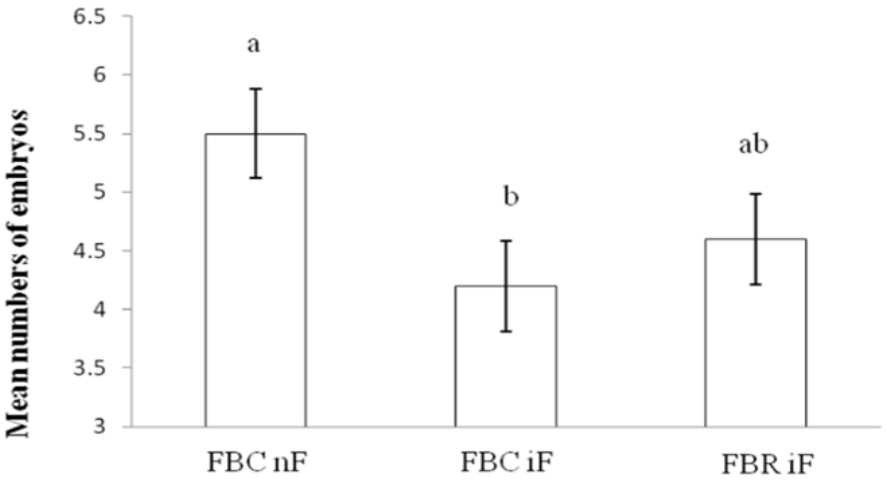

Figure 43. *G. minus* mean number of embryos per female from 2010 – 2013 for Falling Branch Cave Normal Females (FBC nF), Falling Branch Cave Intersex Females (FBC iF) and Falling Branch Road Intersex Females (FBR iF). There was statistical significance between FBC normal (a) and FBC iF (b) intersex females mean number of embryos.

Yearly means (2011 – 2013) of ovigerous females (normal versus intersex) analyzed using ANCOVA (controlling for size), resulted in a significant difference between FBR intersex females and FBC normal females for all 2011, 2012, and 2013 (ANCOVA, General Linear Model: Minitab 17, d.f. = 2; F =6.31, p = 0.013@ *p-value 0.05)*. Ovigerous females from both springs were summarized by the percent of the total population for each phenotype and spring. In FBC, there were a total of 178 (23.28%) normal females and 228 (28.5%) intersexed females with embryos/broods for a total of 406 females collected bimonthly from 2010 – 2013. In FBR there were 377 (46.44%) intersexed females with embryos/broods. There was a significant difference between FBC ovigerous females (normal and intersexed) and FBR intersexed females (ANOVA: General Linear Model: df = 2; F = 20.70; p = 0.001 @ *p-value 0.05*).

Experiment 1: Male choice for phenotype (controlling for size).

Of the 40 replicates, males from both trials (FBC and FBR) preferentially chose intersexed females over normal females (Table 20); however the Chi-squared test (pooled data) revealed this was not significant (Chi Squared = 3.33, df = 1 and p = 0.068 @ *p-value 0.05*).

Table 20. Results of pairings between *G. minus* males and females with regard to female phenotype of both springs combined. Regardless of male spring origin, both males selected intersexed females 70 of 100% over normal females. Males chose intersexed females over normal but it was not significant (Chi Squared = 3.33, df = 1 and p = 0.068 @ p-value 0.05).

Males	**Intersexed Females**	**Normal Females**	**Totals**
Male (A)	15	5	20
Male (B)	13	7	20
Totals	28 (70%)	12 (30%)	40

Source of G. minus FBR (A); FBC (B); Intersex females (FBR and FBC); Normal females (FBC)

Experiment 2 (Part I) Male choice and spring origin

When given the choice, neither FBC nor FBR males showed a preference for FBC or FBR females (FBC: Chi Squared = 0.404; p = 0.525; df = 1; FBR: Chi Squared = 0.1002, p = 0.752; df = 1 *@ p-value 0.05*). Although there were no significant differences in choice, males did tend to choose females from their respective springs more often. (Table 21).

Table 21. FBC and FBR males repaired with females with reference to female spring origin. Males chose females from their respective streams most often, however it was not significant (FBC: Chi Squared = 0.404; p = 0.525; df = 1; FBR: Chi Squared = 0.1002, p = 0.752; df = 1 *@ p-value 0.05*).

Category	**FBC female**	**FBR female**	**Total**
FBC male	12 (60%)	8 (40%)	20 (100%)
FBR male	9 (45%)	11(55%)	20 (100%)
Total	21	19	40

Experiment 2 (Part II) Male choice regarding phenotype for each spring separately (FBC versus FBR).

FBC males chose intersexed females 75% of the time and normal females 25% of the time; FBR males chose intersexed females 65% of the time and normal females 35% of the time (Table 20). Despite the preference again for the intersex specimens (65-75%) a Chi-squared test of frequency of amplexed pairs was not significant; (FBC: Chi Squared = 2.667; p = 0.102; df = 1 and FBR: Chi Squared = 0.90207; p = 0.3373; df = 1; *@ p-value 0.05*, respectively). I failed to reject the null hypothesis and concluded that the male's choice was not dependent upon the phenotype of the female; however, while not statistically significant, there was a propensity for the male to pair with the intersexed females within each spring (Table 22).

Table 22. Re-pairing results for males that chose females within springs (FBC and FBR) with regard to phenotype. Chi-squared test of frequency of amplexed pairs was not significant; (FBC: Chi Squared = 2.667; p = 0.102; df = 1 and FBR: Chi Squared = 0.90207; p = 0.3373; df = 1; *@ p-value 0.05*, respectively).

Male	**Intersexed Female**	**Normal Female**	**Totals**
FBC male	15 (75%)	5 (25%)	20 (100%)
FBR male	13 (65%)	7 (35%)	20 (100%)

Source of G. minus: intersex females (FBR and FBC) and normal females (FBC)

Experiment 3: Re-pairing time for FBR and FBC regarding phenotype (controlling for size).

Twenty pairs from FBR and 40 pairs from FBC were analyzed utilizing ANCOVA to determine if there was significance between the times to re-pair based on females phenotypes (intersexed versus normal). FBR males and intersexed females took an average of 325 seconds to repair; FBC males and intersexed females average repairing time was 233 seconds, and FBC males and normal females average repairing time was 178 seconds. The ANCOVA revealed that there was no significant difference between FBC males and intersexed females, and FBR males and intersexed females, however, there was a significant difference in the repairing times between FBC normal females and FBR intersexed females, (ANCOVA: Minitab 17: df = 2; F = 7.17 p = 0.002 *@ p-value 0.05*). FBR intersex females took a significantly longer time (325 sec) to repair than FBC normal females (178sec). The covariate (female body length) had no effect on timing and re-pairing for any groups (ANCOVA: Minitab 17: df = 2; F = 0.62 p = 0.52 *@ p-value 0.05*) (Figure 44).

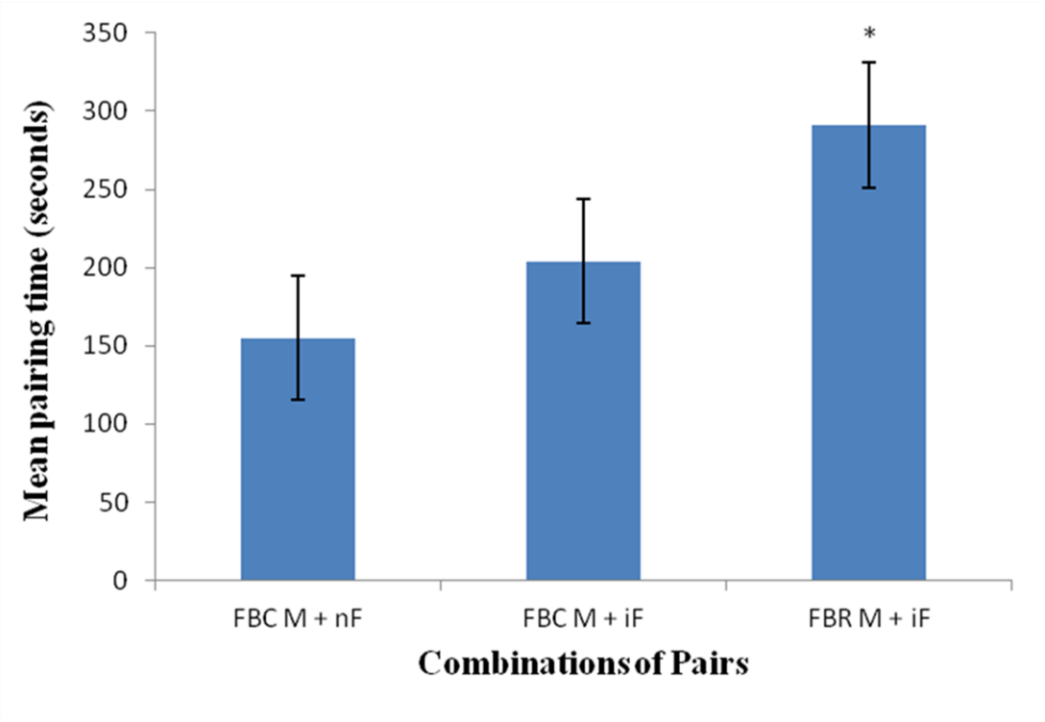

Figure 44. *G. minus* repairing time in seconds for Falling Branch Cave intersex and normal females and Falling Branch Road intersex females. FBC intersex females mean repairing time (204 sec), FBR intersex females (291 sec) and FBC normal females (155 sec). FBR intersex females took significantly more time to repair than FBC normal females at *p-value 0.05* (ANCOVA: Minitab 17: df = 2; F = 7.17, 1 SD, p = 0.002 *@ p-value 0.05*). *Significance *@ p-value 0.05*.

FBC normal female mean length was 5.18 mm; FBC intersexed female mean length was 5.92 mm and FBR intersexed female mean length was 6.60 mm (Table 21).

Table 23. Mean re-pairing timed results for FBC and FBR males and females and female mean lengths.

Spring & Phenotype Groups1, 2, 3	**Mean Time (seconds) ± SD**	**Mean Female Length (mm)**
1FBC Pairs M, iF	233 ± 86.11	5.92
2FBC Pairs M, nF	178 ± 113.55	5.18
3FBR Pairs M, iF	*325 ± 136.78	6.60

During the field sampling, amplexed pairs were observed and recorded in situ (Table 23). The classifications were as follows: numbers of males that were larger than the female, and numbers of males that were smaller than the female.

Table 24. In the field, *G. minus* amplexed pairs collected from FBC and FBR. Male and female sizes observed as either male larger than female or female larger than male. In all amplexed pairs, males were larger than the female.

Stream	Male Size (larger than female)	Female Size (larger than male)	Total
FBC	20	0	20
FBR	20	0	20
Totals	40	0	40

Of the total 40 pairs, all of the males were larger than the females.

Experiment 5: In the laboratory, FBC and FBR male choice and female body length. Large males were characterized as having body lengths greater than or equal to 7.5 mm and small males were less than 7.5 mm. Large females were characterized as greater than or equal to 5.5 mm and small females were less than 5.5 mm. Of the 20 large males, 18 paired with large females and two small females (5.2 and 5.4); of the 20 small males, 16 paired with small females, zero with large females and four with no females.

Table 24. Large males (≥ 7.5 mm) and small males (< 7.5 mm) mate choice with regard to female size (large females ≥ 5.5 mm and small females < 5.5 mm). The choice of large males for large females was significantly greater than the choice for small females and was dependent upon the group; the choice of small males for small females was significantly greater than the choice for large females and was also dependent upon the group (Chi Squared = 32.899, d.f. = 2, p = 0.001@ *p-value 0.05*).

Female Size	**Large male ≥ 7.5 mm**	**Small Male < 7.5 mm**	**Totals**
Large ≥ 5.5 mm	18 (90%)	0	18
Small < 5.5 mm	2 (11%)	16 (80%)	18
No Selection	0	4 (20%)	4
Totals	20	20	40

The choice of large males for large females was significantly greater than the choice for small females and was dependent upon the group; the choice of small males for small females was significantly greater than the choice for large females and was also dependent upon the group (Chi Squared = 32.899, d.f. = 2, P = 0.001@ *p-value 0.05*). Four small males made zero choice for either small or large females and were independent variables (Table 24).

Small male choice for small females was significantly greater than small male choice for large females (ANOVA: F-Value = 26.66, p = 0.001@ *p-value 0.05* and Tukey Test). Four males did not pair, 16/20 (80%).

5.4 Discussion

A significant difference was observed in the mean numbers of embryos between the intersexed and normal females within FBC. This result supports my hypothesis that there was a significantly greater number of embryos brooded by normal females than by the intersexed females, in FBC only. The intersex females had 29.3% fewer embryos than normal females. There are several reasons believed to affect the embryonic differences between normal and intersex females, 1) there may be less room in the ovary due to testicular tissues in intersexes, 2) the AG may be secreting hormones that suppress the ovary, 3) some males have been shown to reduce sperm allocation to intersexes, and 4) intersex females may have deformed brood plates and setae that may lose embryos (Dunn et al., 1993, Taketoni and Nishikawa, 1996; Ford et al., 2003; Glazier et al., 2012). In contrast, Glazier et al., (2012) found that while intersex females tended to brood slightly fewer, but larger embryos than normal females, these differences were not significant overall. The difference in the two studies may be the result of sample sizes, as this thesis looked at three years worth of data and the Glazier et al., (2012) study looked at approximately three months. Supporting the significant result are two studies that have correlated reproductive costs (i.e. lower fecundity and fertility) and intersexed amphipods (Ford et al., 2004; Dunn et al., 1993). Reduced fecundity was also observed in *E. marinus* by 20% in the intersexes over normal phenotypes (Ford et al., 2003). The causes of reduced fecundity in intersexes occurs for a variety of reasons, for example, the androgenic gland may be secreting hormones that are known to suppress ovarian development (Taketoni and Nishikawa, 1996), intersex females may possess testicular tissues (Dunn et al., 1993) and intersexes may lose eggs by "accident" or active ejection from the marsupium (Ford et al., 2003). The second hypothesis was put forth to test the theory of sex-allocation and reproductive fitness between brooding intersex and normal females for both FBC and FBR Springs.

Falling Branch Cave and Road Springs both indicate that intersex females and normal females show similar offspring production, which is further corroborated by other populations of *G. minus* (Glazier et al., 2012). Pennsylvania, USA *G. minus* populations has negligible (< 1%) numbers of intersexes (Ford and Glazier, 2008) and apart from body lengths, the brood numbers and embryonic masses were all in

comparable range of the Pennsylvania populations (Glazier, 1999; Glazier et al., 2012). Interestingly, animals from two freshwater and two marine water systems differ in reproductive output between normal and intersex females. The marine water systems with the following animals (*G. duebeni* and *E. marinus*) have what is considered low numbers of intersexes (0.5 – 9.0%) yet both showed significantly reduced reproductive output, while freshwater (*G. fossarum* and *G. minus*) do not (Kelly et al., 2004; Ford et al., 2003; Jungmann et al., 2004; Ladewig et al., 2007; Ford and Glazier 2008, respectively). Based upon the results of these two analyses the intersexed condition does not prove to be overly costly to the reproductive success of these two populations, thus far.

Two experiments regarding (1) male choice and female phenotype and (2) male choice and female spring of origin did not have any significant affects on the behavior (i.e. choice) in mating. For both outcomes, I accepted the hypothesis that intersexed females would not be discriminated against for the selection of normal females. FBC males chose intersexes 70% and normal females 30% of the time; FBR chose intersexes 65% and normal females 35% of the time. This result is supported by Ladewig et al., (2007); phenotype did not play a role in male choice. The study of *G.fossarum* by Ladewig et al., (2007) investigated the affect of intersexuality on precopula and fecundity, which concluded that intersexes do not negatively affect reproductive success in the studied populations. Moreover, for the result of male choice and spring origin (males chose females from their own springs regardless of phenotype), I would propose that sexual dimorphism may be the overriding factor, and the choice was dependent upon female body size. The male choice experiment between phenotypes (normal and intersex females) was designed to test for phenotype choice, and did not take female size into account. In experiment number one (phenotype male choice) was designed around the sexual dimorphism hypothesis.

Sexual dimorphism, which has been shown as a mechanical constraint and not intrasexual competition for mates, was supported with a series of experiments conducted by Adams and Greenwood (1983). As shown in Chapter 3, intersex females mean body lengths are greater than normal mean female body lengths; perhaps, the size differences have played more of a role than realized and the intersex phenotype is not selected against. Studies have shown that when given the choice,

males select larger and more fecund females than smaller females (Birkhead and Clarkson, 1980). Consider that if in these populations intersex females are the "new normal", it could go a long way in explaining the success of these populations. If males disregard phenotype in favor of a larger female, maybe it is because they do not recognize the difference in phenotype, only size. So, therefore, fecundity would not play a strong role either, as intersex females produce smaller numbers of embryos than normal females. There must be some other underlying factor that is imposed onto the males' decision because the body length of the female and male in precopular is very important for the successful reproduction, especially in freshwater lotic streams.

The cause for intersexes to take significantly more time to repair than the normal females may be surmised as possibly a hormonal influence since size was not a factor in this experiment. It may be that intersexed females do not release enough hormones for the male to become interested again, or possibly they do not regenerate the hormone as quickly as the normal females. Female chemical cues probably play a significant role and males might be able to detect impending female's moult through the release of the hormone (EDCysone/20-OH-EDCysone) (Cornet et al., 2011; Dahl et al., 1970). According to the study of *G. pulex* by Cornet et al., (2011) the moult time may be the key to understanding the decision to mate for both males and females, thereby possibly giving cause to why males took longer to repair with intersexes.

Predicted for this experiment was that normal females would repair more quickly than intersexes and it was confirmed. The possibility for the intersexes lag relates to moult and competition; for example, if after the pairs were separated and female moult stage was early or was not a precopular moult, it could be that the stage of female molt was not conducive to repair without male/female competition (Ward, 1983). Most females become receptive during late intermoult/early premoult stages (Cornet et al., 2011) or when there is substantial competition (Ward, 1986; Cornet et al., 2011). Within each microcosm of this experiment the pairs were not challenged by any other animal (male or female *G. minus*), thus it is possible that the male was a bit more discriminating against the intersexed females. Ward (1983) conducted experiments where the amplexed pairs were separated and then placed back together. The results

of the study showed that within three days all pairs were again amplexed; however there is no mention of intersexed females, so I would surmise that intersexes were not tested. Additionally, as Birkhead and Clarkson (1980) discovered, males will pair based upon molt time rather than the female size. The closer the female is to molting the sooner the male will be able to inseminate her, and so will not need to remain in amplexus as long. The less time the male spends in amplexus, the more energy is conserved and the more time there is to hunt for future females resulting in more DNA distribution. This experiment did not test hormonal levels, sizes of females, or competition of the animals and will be something to consider in the future.

In the field during the March 2018 collections, I recorded whether males were larger than females in amplexus. The categories were large male paired with either small or large female and small male paired with either small or large female. There were no small males paired with females larger than themselves. This finding is supported by all of the previous collections made during this entire investigation from 2010 to 2013, so it was not an unusual finding. Several thoughts of why males select larger females include (1) males must be strong enough to maintain amplexus while swimming with the female in currents (Adams and Greenwood, 1983), (2) males chose larger females because they carry larger broods thereby, increasing their gene pool (Birkhead and Clarkson, 1980) and 3) similarly sized mates are found in the same general area (i.e. patches) as a function of spatial heterogeneity with the size of the stream bed substrate; thus, as long as the size ratio (male/female =1.3 or males 30% longer) favors males, it does not appear that intersexes are at a disadvantage even though they carry smaller broods in the Falling Branch populations (Miller and Buikema, 1977; Ford and Glazier, 2008; Glazier et al., 2012). In addition, experiments conducted by Birkhead and Clarkson (1980) on *G. pulex* pairs, revealed that size really didn't matter when females were within 12 hours of molt; their conclusion was that any sized male could amplex and inseminate any sized female.

When large males were placed with a choice for small or large females, large females were chosen 90% of the time (significant @ *p-value 0.05*) and small females 10% of the time. In FBC and FBR populations, intersex females are larger in body length than normal females; but contrary to other populations (i.e. *G. pulex*) their embryos are larger in mass but smaller in numbers (Glazier et al., 2012). The embryonic size

of intersex females may be a trade-off for fewer numbers of embryos. *G. pulex* show a strong positive correlation between female body length and the number of eggs (Birkhead and Clarkson, 1980), which is a factor in size assortative mating theory. The result is no surprise and supports the hypothesis that males chose larger females over smaller. Within this experiment *G. minus* males have conformed to the same behavioral patterns of other Gammarids (Cheng, 1942; Berg, 1948; Birkhead and Clarkson, 1980; Bollache and Cezilly, 2004). However, while other Gammarids preferred normal over intersex females regardless of size, FBC and FBR did not.

6. General discussion

OBJECTIVE: This Chapter intends to review and comment on the scientific methods adopted by the work, discuss the main findings, and suggest directions of future work.

The overall objective of this study was to increase the knowledge base of the two populations of *G. minus* (Say, 1818), found in the Falling Branch Road area. The primary aim was to explore the amphipods female intersexuality phenomenon, along with several subsidiary aims 1) document the biology and ecology of both sample sites, 2) investigate population dynamics for sex ratios (males, females, and intersexes) of both populations and investigate population dynamics of FBC for a long-term (1977 – 2013) comparison with two other studies (Miller, 1977 and Haley, 1997) 3) compare and analyze body lengths of males, females, and intersexes of both populations, 4) compare and contrast mating cycles and fecundity of normal females with intersexed females for both populations, 5) explore general cause(s) of intersexuality for both populations, 6) investigate surrounding populations of *G. minus* for the intersexed condition (Virginia and Ohio), 7) determine male selection preferences between normal versus intersex females and 8) observe amphipod behaviors during various pre-copula stages (female small body length versus female large body lengths and small male body lengths versus large male body lengths).

G. minus, a well studied amphipod in the United States is found throughout the Appalachian Mountain Range. The mountain range is characterized as karst topography, where alkaline freshwater streams and underground cave spring-runs are prevalent (Chapter 2). The organisms' life history has been extensively studied and a substantial measure of data has been accumulated. The knowledge of the organism regarding population structure, maturation and reproductive cycles, female fecundity, as well as, physiology and physical attributes have all been previously characterized (Miller, 1977; Haley, 1997, Buikema, 1980; Glazier, 2009, Glazier and Ford, 2008; Glazier et al., 2012).

This study was conducted to investigate the two populations of the Falling Branch Road area, in order to fill in the gaps of missing information (i.e. sex determination, causes and costs of intersexuality, and reproductive behavior).

The investigation of the Falling Branch Road area occurred over a three year period (2010 – 2013), which was a long-term study that included both field observations and laboratory experiments. The sampling protocol was partly determined by the distance to the sample site, and work schedule. Therefore, bimonthly sampling for the three year period of both FBC and FBR *G. minus* amphipods was agreed upon. A total of 17 months were sampled that extended across all four seasons (summer, winter, spring, and autumn) of both locations (FBR and FBC). All of the data collected would serve as the primary source for the statistical and observational analyses for this thesis.

The focus to determine the cause(s) of intersexuality in these extraordinary populations was put forth as a priority of this research. The literature supports that the prevalence of intersexes in amphipod populations may vary by species, location and if known, causes (Dunn et al., 1990, 1993; Kelly et al., 2004; Hough et al., 1992; Ford et al., 2004; Ladewig et al., (2004). Considering that in most cases, the prevalence of intersexuality is minimal (Dunn et al., 1990; Ladewig et al., 2002 and Barbeau & Grecian, 2003; Ford and Fernandes, 2005) the high prevalence of intersexuality of FBC and FBR females is therefore, extraordinary (Miller, 1977; Buikema, 1980; Ford and Glazier, 2008; and Glazier et al., 2012). In fact, these *G. minus* populations have the highest prevalence of intersexuality ever recorded in an amphipod species, FBR females were 100% intersex and no normal females, while the intersex females in FBC ranged from 60 to 75% over the course of this study (Chapter 3). This assertion is supported by the evidence of many other investigations (Sars, 1895; E. O. Sexton, 1906 and 1924; Tattersall, 1910; Sexton and Huxley, 1921; Buikemia, 1930; Buikemia and Miller, 1977; Dunn et al, 1990; Ladewig et al., 2003; Ford and Fernandes 2005; Ford and Glazier, 2008; and Glazier, et al., 2012), as well as, the comparison of three other springs within the surrounding area (Bradley Run, Cedar Fork Cave and Antietam Creek) that were personally examined (Chapter 3).

FBC and FBR populations were at odds with regard to Charnovs' sex allocation theory. Neither population followed the predicted sex ratio of 1:1 (M: F) for gonochoristic animals, as described by Charnov (1982) and Fisher (1930) (Chapter 3).

The incidence of intersex females were significantly greater than normal females (Chapter 3) and the incidence of gravid intersex females was highly correlative between springs. It may be that seasonal breeding could possibly make up for the reproductive costs of reduced numbers of eggs carried by intersex females. Moreover, this seasonal breeding pattern may also be under parasitic control. There is evidence that microsporidia are temperature sensitive. Kelly et al., (2002) demonstrated that temperature had a significant effect on both transmission and feminization efficiency. Feminization of *G. duebeni* males was 85% efficient at 10^{o}C while at 5^{o}C efficiency was 49%. Based on the evidence, we may predict that stream temperatures of FBC and FBR may have an effect on the seasonal patterns of the parasite transmission and feminization of males. The similar growth and maturation patterns were instrumental in other patterns related to reproduction. Reproductive success is dependent upon male and female amphipod sized relationships (Naylor et al. 1988; Watt and Adams 1994), which enable mate-guarding (Hatcher and Dunn, 1997). The growth patterns for the intersex females of both populations present an obvious pattern, while normal females from FBC did not (Chapter 3). As such, similar maturation patterns indicated similar precopular, breeding and fecundity cycles between the two intersex female populations (Chapter 3). The growth traits of intersex females (i.e. delayed maturation and larger individuals) may offset predation of normal females and so, the reproductive costs ensued by female intersexuality may be worthwhile. In addition, if stream temperatures influence feminization of these populations (as discussed above) then the cooler temperatures during late autumn into winter, would support a temperature driven pattern of similarity (Kelly et al., 2002).

Sex ratios and prevalence of intersexuality of FBC and FBR were determined to be persistent with the previous documentation as far back as 1977 (Miller). In FBR the prevalence of the female intersex condition remained consistent at 100% (Chapter 3), in FBC, Miller (1977) originally put forth that 100% of females were intersexed (Miller, 1977), but since the Miller (1977) study, intersex females have declined. This study showed FBC intersex females had varied over the 3-year time span

vacillating between 63 – 75% (Chapter 3) of the total female population. The lower end of the proportion of intersex females may be due to lower numbers of total specimens collected. Thus, in the Buikema (1980) study of FBC, a 60% intersex female population was recorded, due to the proportion of females with papillae decreasing. If Miller (1977) was correct about all females displaying the intersex condition then perhaps we can say that this decline has occurred from 1980 onward. While a definitive cause of the intersex condition for FBC and FBR has not been determined, several possibilities warranted further study (environmental Chapter 3 and parasitic Chapter 4).

The environmental measurements for FBC were not significantly correlated with sex ratios or mean body lengths of normal females. In contrast, the following associations between environmental parameters and intersex females were correlated linearly, 1) increased pH and an increased incidence of female intersexuality 2) increased alkalinity and increased mean body lengths 3) there were only weak correlations between water temperatures, dissolved oxygen, alkalinity and hardness regarding intersex prevalence and/or mean body lengths.

Regarding FBR, there were no significant correlations but only one moderate correlation between mean body lengths and stream temperatures. This correlation was negative denoting that as the mean water temperatures rose, mean body lengths of intersex females decreased (Chapter 3).

In amphipods, other studies have demonstrated that environmental sex determination (ESD) exists (Dunn et al., 2005; Guler, 2012). In this study, FBC intersexuality is linked to photoperiod. During sampling events from 1980 - 2013, both pH and the occurrence of intersex females decreased, but since there were only 3 points of measurements available (Buikema, 1980, Haley, 1986, and this study) a correlation cannot be assumed. The mean pH in FBR from 2010 to 2013 was 7.7, and female intersexuality remained 100%. If the pH value and prevalence of female intersexuality are interrelated, then intersex animals may be a biological indicator of an unknown influence. The Buikema (1980) study supports my theory that an environmental factor (i.e pH) may be an indicator that EDC's are the cause of such a

high incidence of intersexed females. The occurrence of an EDC affecting these populations cannot be ruled out.

Within the FBR population, normal females were never found despite observing 1348 specimens. All females (100%) displayed the intersex condition and remained at that prevalence over the three year period. Considering the 100% female intersexuality was reported as far back as the 1970s and at several periods in between this study one might speculate that the condition has not changed (Miller, 1977; Buikema, 1980; Glazier et al., 2012). As with the FBC population, the sex ratio was male biased, which is also unusual amongst amphipods, but was characterized by peaks and lolls, which may be linked to temperature driven parasite prevalence, as discussed above. The female population peaked during the winter months (Nov – Feb) and males during the autumn (Sep – Nov). Amphipods engage in a unique reproductive behavior (amplexus); therefore, it is necessary for the males to be larger than females (dimorphism) to employ this behavior. Considering FBR is under a discontinuous breeding cycle, it would make sense that the males numbers would peak before females to allow adequate time for maturation and growth.

In FBR, males and intersex females were both significantly correlated with photoperiod (Chapter 3). From early winter to summer sex ratios between intersex female proportions were negatively correlated with males; intersex females increased (28 – 34%) as males declined (68 – 52%); accordingly, when day light lessened intersex females increased. Dunn et al., (1993), showed that photoperiod and stream temperature were linked to the intersex condition. Interestingly, in FBR, both male and female phenotypes' were significantly correlated to photoperiod and intersex females were further correlated to stream temperatures. This could be a possible synergistic effect. Moreover, in FBC intersex females were also significantly correlated with photoperiod (Chapter 3). Still, both populations maintain different sex ratios but, photoperiods are the same. It had been suggested that cave populations may be interbreeding with surface populations (Fong, per comm., 2012), however, neither FBC nor FBR populations exhibit morphological characteristics of a cave specimen, as described by Culver (1995). To consider photoperiod, the sex ratios would likely fluctuate (Buikema et al., 1980), and they do not.

The reproductive cycles for normal females in FBC were continuous for all twelve months of each year and are quite possibly multivoltine (Sayed et al., 2016). Intriguingly, FBC and FBR intersex females maintained a correlation during growth and maturation, which peaked from autumn to winter of each year indicating a univoltine annual reproduction cycle (Sayed et a., 2016) (Chapter 3). Further analysis of the relationship between springs and intersex females revealed a significant pattern, during winter months, (Chapter 3).

The following summarized points are summary items for consideration as to the causes of intersexuality in these populations, and a possible source of the decreased sex ratio (i.e. intersexed females' decline of 35 – 40% since 1977) within FBC, as well as, other trends between FBC and FBR.

- The pH and percentage of intersexed females in FBC have both decreased from 1977 to 2013.

- Alkalinity correlated with FBCs' normal female and FBR intersex female body lengths. Both were negatively correlated as alkalinity increased, body lengths decreased.

- Growth during the Winter season (Dec., Jan., Feb.) was similar between both FBC and FBR intersexed females (i.e. maturation patterns and incidence of ovigerous females).

- FBC intersexed females were positively correlated with stream temperatures; as temperatures increase, intersex female to normal females ratio increase.

- Photoperiod significantly correlated with FBR males and intersex females; long day - % of males was greater and short day - % of intersex females was greater.

- Photoperiod significantly correlated with FBC intersex females; long day - % intersex females were greater, and males were positively correlated with photoperiod. Normal females were negatively correlated with photoperiod; as day light increased, the % of normal females decreased.

- The percentage of ovigerous females was correlated between alkalinity and FBC normal females and FBR intersex females; as alkalinity increased FBC normal females increased and FBR intersex females decreased. Seasonally, FBR intersex

females decreased with an increase in DO.

Based on personal observation that FBC is always more densely populated than FBR, I would propose that FBC is a more favorable and stable environment for the animals than FBR. The higher density of animals in FBC is probably due, in part; to higher alkalinity, hardness and concentrations of Ca^{2+} and Mg^{2+} that provide more ion availability to amphipods (Glazier, 1992).

In Chapter 4, causes of intersexuality were investigated. It was anticipated that a high prevalence of feminizing parasites would be involved in the intersex females' populations (Ford and Glazier, 2008; Glazier et al., 2012; Ginsburger-Voel and Desportes 1979; Dunn et al., 2001; Terry et al., 2004; Werren et al., 2008; Engelstaedter and Hurst, 2009; Cordaux et al., 2001; Short et al., 2012 Dunn and Smith, 1993, 1996, 2001; Dunn et al., 1995; Kelly et al., 2004). For the first time in these populations (FBC and FBR) a novel microsporidian parasite was found (Chapter 4). These populations have never been found harbouring parasites (Buikema, 1980; Ford and Glazier, 2008). The histology methods were not as revealing as hoped for due to the number of specimens that were negative. Comparatively speaking, there were 400 animals that were screened with the PCR method and 20 with histology. The differences in the sample size possibly have an effect on this outcome. The incidence, as shown from the histology slides resulted in a low prevalence of visual spores observed in the muscle tissues of the gonad area (histology slide for FBC intersex female 1.57%). The low number of positive slides and the high number of negative slides was not surprising considering the results for previously examined specimens by Buikema (1980) and Ford and Glazier (2008). Quite possibly, histology may not be the best method to detect microsporidia, as they are intracellular parasites, which are very small. Another reason may be due to the use of the entire organism for the histology sample and estimating the sectioning of the gonad area. The female's gonads are located from approximately the 2^{nd} to 5^{th} segments; however the area dissected for histology ranged from the 4^{th} to the 8^{th} segment. In this study, ten slices per 2 – 3mm of tissue (@ 0.5µm each) was adequate to reveal the presence of a parasite, but was not thoroughly representative of the area. Sliced tissue sized at 0.5µm each is adequate for most applications; however in an area of 2 – 3mm of tissue it may not have been the best, considering a large portion of tissue was missed.

Therefore, the percentage of positively infected amphipods may be much higher. In addition, the location (4^{th} to 8^{th} segment) that was used for the histology may not have been precise, as parasites may be found in the total reproductive tract, particularly ovum (Ford et al., 2005). Dissection of the female could be improved if the following methods from Ford et al., (2005) were applied to future applications; all appendages (plus gills and coxal plates) removed, the cuticle cut ventrally along the midline, the hepatopancreas and intestine removed so the actual gonads can then be observed in situ. The method of dissection utilized in Ford et al. (2005), allowed for an increase in specificity, which ensured an easier task of identifying gonad morphology. Overall, better processing of tissues, whether for SEM or histology, would greatly enhance the quality of the slides in this study. It would also be of benefit to substantially increase the number of slides for the given area to ensure a more thorough examination of the gonads.

As discussed in Chapter 4, PCR analysis resulted in low prevalence of infection for both FBC (10%) and FBR (11%) *Microsporidia sp*. however the resulting proportions may not be truly representative of the actual parasitic infection. There were some problems with PCR reactions, thus it quite probable that the prevalence of *Microsporidia sp.* or even another parasitic species occurrence could be much higher. A significantly higher incidence of microsporidian infections was observed in the intersex female specimens than the normal males and females which recorded zero infection. Even at such a low occurrence this still suggest the parasite might be feminizing and that intersexuality could be occurring through the incomplete conversion of males to females, as proposed by Bulnheim (1965). A female-biased population is a known consequence of feminizing parasites; therefore, even if there are high infections of feminizing parasites the male-biased sex ratio would still be highly unusual (Dunn et al., 1995; Terry et al., 2004).

The occurrence of male feminizing parasites would possibly explain the oddity of the male biased sex ratios (Ford and Glazier, 2008), only if these populations were originally all male. In the scenario that these are all male populations, the presence of feminizing parasites (with the ability to manipulate males to phenotypic females) would perhaps be necessary to continue the reproductive processes that maintain success of the populations, otherwise, according to the theories of sexual allocation

(Charvnov, 1982) the populations would go extinct. Other theories considered for these population structures (e.g. male-biased and female intersexuality) include geographical separation and, genetic drift.

Supposing that both FBC and FBR were geographically separated (i.e land mass removal and deviation of stream flow) from the source population, the isolated population would strive to become stabilized. For example, if one sex is more costly to produce than the other sex, sex-allocation theory would predict that the population sex ratio (at equilibrium) is biased towards the cheaper sex (Fisher, 1930; Charnov, 1982). Hence, in FBC and FBR, the male biased sex ratio could be interpreted, as the male is the less costly sex to produce.

On the genetic level, isolated populations are subject to low or asymmetric gene flow, resulting in reduced allelic richness and heterozygosity. Considering genetic drift and population bottlenecking might be the most logical. As described above, when a population becomes isolated it is also a greatly decreased allele pool that can significantly effects future generations. The amphipod populations in FBC and FBR are sexually reproducing animals that are probably geographically isolated, thus genetic exchange between males and females may have eventually increased or decreased alleles. Small populations will likely result in fixed genes, and genetic homozygosity (Carlini et al., 2009). If genetic drift did occur, this could possibly explain the male-biased sex ratios for both populations, and a fixed gene resulting in feminized males. In FBR, the intersexed female population has consistently been 100% since the initial recording of the stream (Miller and Buikema, 1977), therefore, a good candidate for the fixed gene theory.

The retrieved microsporidian 18S rDNA was 97% related to a *Microsporidia sp*. found in the amphipod *Micruropus wahlii*, an endemic species of the ancient fresh water lake, Lake Baikal, Russia (Madyarova et al., 2015). The microsporidian sp. fell within the same clade as Microsporidia sp. BWOH10 (FJ756195.1) and shared the most common ancestor. The large clade included the following families (Microsporidea, Nosemadidea, Encephalitozoonidea, Pleisophoridea), which have shared ancesteral characters with the unknown species including the feminizing parasites *D. duebenum* and *Nosema granulosis* (Chapter 4). If feminizing parasites

are the causes of this anomaly it may have occurred at an earlier age, which is unknown because this study only looked at adults.

It was hypothesized that intersex females suffer a costs to fecundity and fertility that results in smaller broods, less embryos and poor fertility (Dunn et al., 1993, Taketoni and Nishikawa, 1996; Ford et al., 2003; Glazier et al., 2012). Within this thesis, observation of female fecundity and behavioral experiments were conducted to test these assumptions. A significant difference was observed in the mean numbers of embryos between the intersexed and normal females within FBC. The intersex females had 29.3% fewer embryos than normal females. Supporting the significant result are several studies that have correlated reproductive costs (i.e. lower fecundity and fertility) and intersexed amphipods (Ford et al., 2004; Dunn et al., 1993).

In Chapter 5, mean numbers of embryos carried by normal females was significantly higher than the embryos carried by intersex females. Many studies support this outcome and define it as one of many costs of intersexuality (Ford et al., 2003, 2004; Jormalainen, 1997; and Ladewig, 2002). However, considering that this is a cost to various populations of amphipods it does not appear to be detrimental to the Falling Branch Road area amphipods, considering the densities and reproductive health. Intersex females have been found to have larger (mass) embryos than normal females (Glazier et al., 2012), suggesting that despite the lower eggs per brood, the parental investment in terms of energy per brood does not differ.

The mate choice experiment between reproductively receptive normal and intersex females and males revealed that males did not discriminate against the intersex females (Chapter 5). Rather, the males result indicated the choice was based in size, and possibly receptivity not phenotypes. It has been documented that larger normal female amphipods yield more embryos than smaller normal females (Ford et al., 2003; Glazier et al., 2012) thus; males have been shown to choose larger females based upon the advantages in fecundity. Consequently, in the FBC and FBR populations, perhaps size does matter. In Chapter 3, mean sizes were statistically evaluated and within both populations males were larger than intersex females and intersex females were larger than normal females (all were significant). Considering the lineage of intersexuality of each population, males may have simply adapted to intersex females and so, are not as selective.

Choice experiments were conducted regarding male choice in three different scenarios 1) females from the same spring as the male (spring of origin) disregarding size and phenotype 2) female phenotype (disregarding spring and size) and 3) female size (disregarding spring and phenotype) (Chapter 5). The expectations from the experiments were as follows: 1) males would prefer females from their own spring 2) males would prefer normal females over intersex females and 3) large males would prefer larger females over smaller females and small males would prefer smaller females over larger females. In review, males had a propensity towards females from their own spring of origin, but it was not significant; males preferred larger females over smaller females regardless of phenotype; and finally, large males preferred larger females over smaller and small males preferred small females. There were never small male's amplexed with a larger female.

Of all the choice experiments, males selecting large females over phenotype were in conflict with some studies, but not in others. The results of this choice study was supported by Ladewig et al., (2007) who found that male choice was based upon female reciprocity and that males also chose females nearer to molt. Amphipod body size just may be the most important driver of reproduction. Body size is attributed to other outcomes and the inevitable success of the male to mate. The success of the male to mate-guard females is dependent upon the males' ability to control and guard the female in various conditions. Pre-copular behavior, mate-guarding and sexual selection have evolved due to the need for favored characteristics to be passed on to the offspring. In addition, males must protect females and be strong enough to ward off other males (Conlan, 1991; Jormalainen, 1998). This is true particularly in male-biased populations, as rivalry for female's results in vigorous intersexual competition (Jormalainen, 1998).

The re-pairing of males and intersex versus normal females was supported by previous studies in that amplexed pairs of males and intersex females when separated took a significantly longer time (325 sec) to re-pair, than males and normal females (178 sec) (Dunn et al., 1993). This result may indicate a disruption to pheromonal activity (Ford, 2004). Intersex females may be internally intersexes and have a remnant AG that could possibly suppress female pheromones, or produce male

pheromones; as well as, possessing testis, vas deferens, and other internal male sex characteristics. If intersex females do not produce adequate pheromones this could possibly affect the signal of receptivity of the female (Ladewig et al., 2007).

Overall, this study was an in-depth analysis into the populations of *G. minus* and the high prevalence of intersexuality. First, an analysis of population dynamics, which included annual and seasonal sex ratios, maturation and reproductive cycles, comparisons and correlations of the effects of environmental variables on organisms, and correlations and similarities between the two springs (FBC and FBR). Additionally, a long-term (1977 to 2013) evaluation was undertaken, utilizing the data from this study and two other studies, to identify relationships and/or correlations of *G. minus* in FBC (Miller, 1977; Haley, 1997).

The results of this study have identified a consistently high and relatively static rate of female intersexuality for these populations, as well as, a theoretical cause of sex determination. An element of sex determination is likely due to photoperiod. The unknown species of microsporidia that was detected in intersexed females was so low that the theory of male feminization was not conclusive. In theory, the fluctuation of pH in FBC could be an indicator of stream health. If contamination has occurred or is still relevant, and sex determination is polygenic (Bulnheim, 1978) EDC's could positively cause the intersex conditions. Because of the uncertainty of the streams paths, it is not possible to discern any contamination incidents or exposures. It has been demonstrated by Ford (2004) that pollution (EDC's), both directly or indirectly, can be a cause of intersexuality (Ford, 2004). As described in Chapter 1, many pollutants have been shown to be the cause of numerous and diverse affects on organisms specifically reproductive anomalies. Therefore, the influence of such chemicals cannot be ruled out and must be considered a probably cause of the high incidence of female intersexuality.

While many characteristics of FBC and FBR springs amphipods have been brought forth, the cause of intersexuality remains an open question.

6.2 Application of Research and Future Direction

This section is to discuss how the results from this study contribute to the knowledge base of Gammarus minus, and future direction of the study of the *G. minus* populations, specifically in the Falling Branch Road area.

The data gathered in this study was put forth as further knowledge to describe and understand the two populations of *G. minus* found in the Falling Branch Road area. The chosen areas of study were population dynamics regarding growth, maturation and reproductive patterns. These areas were further explored to describe sex ratios, fecundity and fertility of normal females versus intersex females and ultimately the cause(s) of the high prevalence of female intersexuality.

Further studies would appropriately include a breeding experiment to collect data regarding "inheritance" of feminizing parasites (Dunn and Smith, 1993; Dunn et al., 1995). The breeding studies that were attempted for this thesis were not successful as the amphipod was very sensitive to; perhaps, more research into the husbandry of this species and a new environmental design would prove to be fruitful. Breeding studies to elucidate heredity of sex ratios, parasitic infections, and experiments based on ESD (e.g. photoperiod, temperatures, etc.) could possibly help to understand how the AG is functioning in sexual development. During breeding studies, mimicking field conditions and then manipulating these factors to isolate determinants and utilizing endpoints such as sex ratios, intersex females, fecundity and fertility, would be useful.

A solid study using molecular methods to identify and determine the function genetics has in sex determination. In this study, molecular methods were weak, due inexperience in this particular science. Through more direction and guidance in dissection, molecular technique and even primer selection/design would most likely prove advantageous to furthering the knowledge base of these populations.

The high prevalence of female intersexuality provides a unique opportunity to potentially identify more host-parasitic relationships and the effect on individuals and populations as a whole. These populations may even challenge the long standing theory of sex allocation (Charnov, 1982). Considering that population genetics,

ecology in general and reproductive science are all so diverse in invertebrates, understanding how these mechanisms operate could open doors to new pathways of discovery. These unique specimens, utilized as models, would be perfect for studying adaptations, parasitic manipulations, and mechanisms of sex determination. The overall success of diverse groups should inspire.

The overall importance and contribution of this research may be applied towards the plasticity and adaptation qualities of the animal kingdom. The diversity of phenotypes and sexual reproduction of these populations may lead to stronger evidence concerning the successes that challenge sex allocation theories. Adaptation to the environment and other stressors (i.e. evolutionary constraints) are perhaps, the new norm within the Falling Branch Road *G. minus* populations.

This study has some fascinating results, as listed below:

- Highest incidence of intersexuality in amphipods
- Longest prevalence of high intersex incidence in an amphipod population (40+ years)
- First discovery of microsporidia in *Gammarus minus* populations
- First discovery of a potential feminizing parasite outside the Genus *Nosema* and *Dictyeocela*
- Normal males don't have a preference for normal over intersex females
- Intersex females produce approximately 29.3% fewer eggs than normal females
- Males take approximately twice as long to repair to intersex females compared to normal females

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